GENE THERAPY FOR OSTEOSARCOMA:

IN VITRO CHARACTERIZATION OF FIVE TUMOR CELL LINES AND DEVELOPMENT OF TREATMENT MODELS *IN VIVO.*

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by

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ABSTRACT

Four human osteosarcoma cell lines (MNNG, TE85, MG63, and G292) and a human-derived osteosarcoma metastatic to murine lung (MLM) were transduced *in vitro* using retroviral vectors containing the Herpes simplex thymidine kinase (HS-tk), human interleukin 2 (IL2), and ß-galactosidase (BAG) genes. Each osteosarcoma cell line was stably transduced, and the HS-tk gene effectively conferred ganciclovir (GCV) susceptibility to transduced cells. Each cell line showed a bystander effect (whereby wild type tumor cells are killed by co-incubation with HS-tk positive tumor cells and GCV).

MNNG cells were used to develop a series of experiments in athymic nude mice to investigate the utility of gene therapy for the treatment of experimental osteosarcomas. Subcutaneous implants of mixtures of tumor cells and HS-tk vector producer cells (vpc) resulted in the development of tumors which were completely cured upon administration of GCV. Subcutaneous implant of mixtures of transduced and wild-type cells resulted in a potent bystander effect upon administration of GCV, with complete tumor ablation when as little as ten percent of the cells were HS-tk positive. A significant anti-tumor response was seen against primary tumors comprised of unmodified cells when a secondary tumor of transduced cells was induced at a distance of one centimeter, which supports a soluble bystander factor model. The presence of IL2-transduced cells significantly improved the efficacy of treatment, suggesting a synergy between the HS-tk and IL2 gene products. A significant anti-tumor response was seen in the treatment of established osteosarcomas by the injection of HS-tk vpc.

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INTRODUCTION and REVIEW OF THE LITERATURE

Osteosarcoma

Definition and Incidence.[1,2] Osteosarcoma is a primary malignant tumor of bone, deriving from primitive bone-forming mesenchyme and characterized by the production of osteoid tissue or immature bone by the malignant proliferating spindle cell stroma.[3] Osteosarcoma is the most common bone cancer of childhood and adolescence, accounting for 60% of the 5.6 cases per million children, and 20% of all bone tumors. This disease afflicts males more frequently than females, at an 8:5 ratio, but shows little preference for race or geographic location. In the United States, there are about 900 new cases per year.[4]

Osteosarcoma strikes at a peak age range of ten to 25 years, but it has been reported in toddlers (the youngest being 35 months) and the elderly. The peak incidence for females is 13.5 years, and for males is 14.5 years.[2] Because these ages correspond with the typical onset of the adolescent growth spurt, a relationship between rapid bone growth and development of cancer is suggested.[5] Indeed, the greater incidence among males may be related to the larger volume of bone formed during the growth period. Patients with osteosarcoma tend to be tall for their age, typically in the 80th percentile or greater for height.[2]

Histological Subtypes.[6,7,8] Conventional subclassifications of osteosarcomas include four major forms, and at least four rare variants, all of which are microscopically distinct. The osteoblastic subtype, involving malignant bone as the primary neoplasm, is the most common type, accounting for 44% of all osteosarcoma cases. The chondroblastic subtype, first involving malignant cartilage, ranks second with 27% of all cases. An anaplastic subtype is seen in 17% of cases. A fibroblastic subtype, involving malignant stroma, has been identified in nine percent of all cases. About three percent of patients show a histological mix of these types.

Rare variants include parosteal (juxtacortical) and periosteal forms, which are seen in five percent of cases. Both are superficial, arising from the cortex and not involving the bone marrow. These types are more frequently seen in patients over 30 years of age and are reported to be stubborn to treatment but seldom metastatic. Another infrequently observed (three percent) subtype is telangiectatic, which is characterized by giant cells and a cystic tumor. The most rare form (one percent) of the disease (and the

most deadly) is multifocal sclerosing osteosarcoma. This type is only seen in children under ten years, and is invariably fatal. It involves multiple primary tumors in the ends of most long bones, and it tends to disseminate rapidly to the lungs and soft tissues.

Clinical Course [2,9]. The onset of osteosarcoma typically involves pain due to tension placed on the periosteum. The pain is slight and sporadic, and has been compared to a sprain, rheumatism, or arthritis. This vague complaint generally precedes tumor appearance by days to months. The site of the primary neoplasm is variable and has prognostic value. Half of all osteosarcoma cases are seen in the femur; 80% of these are in the distal portion of the bone. The disease is also seen in the mid- and proximal femur, the proximal tibia, and the proximal humerus. Osteosarcoma arises infrequently (15-20% of all cases, but less than ten percent of pediatric cases) in axial bones such as the pelvic bones, vertebrae and ribs. Primary cancer in the jaw is reported to occur in seven percent of cases, and typically displays an aggressive clinical course. Rarely, osteosarcoma arises in the fibula, ulna, radius, metacarpals, and metatarsals. Osteosarcomas of the long bones tend to originate at the metaphysis. Osteosarcomas generally grow very rapidly, with a median doubling time for tumor volume of 34 days.[10]

Metastatic disease.[11] Much of the mortality associated with osteosarcoma is due to metastasis of malignant cells through the blood to distant, often multiple sites. Over 90% of metastasis is to the lungs, and 15-30% is to other bones. Less common sites are the pericardium, kidneys, adrenal glands, and brain. Ten to 20% of patients present with visible macrometastases, and a greater proportion (perhaps 40%) have microscopic metastases. A significant percentage of patients experience metastasis at some point during the course of the disease. The two-year survival rate of metastatic osteosarcoma is 30% or less.

Diagnosis.[2] The external appearance of the area overlying the tumor may vary widely among patients, depending upon the site, progression, and other factors. Typically, the overlying skin will not ulcerate but may be stretched taut and have an increased temperature; the tumor may be palpable, and richly vascular tumors may pulsate. Twenty percent of patients with osteosarcoma will experience a pathological fracture in the affected bone; this is their initial reason for seeing a physician. The classical demonstration of an osteosarcoma is done radiologically; X-ray analysis will show a "sunburst" appearance of radiating osteoid cell production from the primary lesion

to the surrounding soft tissue. Half of patients present with an elevated serum alkaline phosphatase level, which may be of diagnostic significance.

Staging Information[2]. Clinical cases of osteosarcoma are divided into three categories. In stage I, or low grade, osteosarcoma the tumor is localized to the bone of origin; this may represent an early tumor or a less aggressive subtype. Tumors at this stage (and stage II below) are subdivided into intramedullary and extramedullary categories, depending upon the site of development. With the best available therapy, the two-year survival at this stage is 70-90%.

Stage II, or high grade, osteosarcomas involve well-developed or more aggressive tumors. Tumors in this stage remain localized to the bone of origin, but "skip metastases" (smaller, secondary tumor foci) may be present, indicating a worse prognosis.[12]. The two-year survival at this stage is 40-60%.

Stage III, or metastatic osteosarcoma, involves the spread of the cancer through the hematogenous route to distant sites in the body. Ninety percent of metastatic disease is to the lung. These patients have high risk of pulmonary hemorrhage and failure, obstruction of the pulmonary vessels, and other life-threatening conditions. The second most common site of metastasis is to another bone. With the best available therapy, the two-year survival rate at this stage is 30% or less, and the five-year survival rate is generally below 20%. Patients who survive beyond five years often experience no further trouble, in which case they are considered to be cured of the disease.[13]

Prognostic features[1,3,13]. Many parameters have a significant influence on the clinical course of osteosarcoma. The site of the primary tumor has perhaps the greatest predictive importance. Tumors of the limbs have a better prognosis than do tumors of the axial skeleton. Tumors of the metacarpals, metatarsals, and tibia tend to be the most favorable, while tumors of the clavicle, scapula, other nonexpandible bones, and upper half of the femur often have less favorable outcomes. Osteosarcomas of the skull, vertebrae, and pelvis have a very poor prognosis due to the limitations in treatment avenues. Moreover, a distal location of a tumor is more favorable than is a proximal location. Smaller tumors generally have a better prognosis.

The age of the patient is also an important factor; patients between the ages of 20 and 50 typically fare better. Younger patients often develop tumors which are biologically more belligerent, and older patients generally do not respond as well to

treatment. Female patients typically fare better than do male patients. The duration of symptoms prior to treatment has a predictive value; surprisingly, a longer duration of symptoms carries a better prognosis, as high grade tumors are usually less tolerable.

Tumor cell histology is another indicative factor. Periosteal and perosteal types have favorable prognoses. Differences among the other types may be only incidental. It has also been reported that increased tumor cell ploidy may be negatively correlated with the prognosis.[8]

The serum levels of at least two enzymes have a significant predictive value[13]. Patients presenting with elevated LDH (lactic dehydrogenase) levels have a long term survival rate which is reduced by 50% compared to patients with low LDH levels. Elevated alkaline phosphatase levels indicate a greater risk of metastasis. The presence of the HLA-A3 haplotype has been associated with a poor prognosis.[14] The prognosis for metastatic disease depends upon a number of variables, including surgical resectability, number of lung nodules (three or fewer are favorable), and recurrence frequency.[15,16]

Etiology.[2] The cause of osteosarcoma remains unknown, but multiple factors may be involved. Neoplasms in long bones tend to arise near the growth plate, where the greatest increase in length and size occurs. Thus, random aberrations of the normal process of bone growth and remodeling may contribute to the onset of osteosarcoma. The correspondence of cases with the adolescent growth spurt suggests that changing hormone levels may have an influence.[17]

Mounting evidence strongly implicates a genetic basis for the disease.[18] Before molecular analysis was available, multiple cases of osteosarcoma within families were diagnosed at a higher level than dictated by chance, and sixteen sets of siblings with the disease have been reported.[19] More recently, allele losses at specific loci of transformed cells have been identified,[20] and many of these loci correspond to the sites of tumor suppressor genes. Specifically, a deletion along the short arm of chromosome 3 is seen in 75% of osteosarcoma patients. A deletion along the short arm of chromosome 13 (corresponding to the RB1 tumor suppresser gene) is seen in 68% of patients. In the osteosarcomas of 72% of patients, a loss along the long arm of chromosome 17 (corresponding to the p53 tumor suppresser gene[21]) is observed; loss at this loci is prognostic of early lung metastasis.[22] Finally, 64% of patients show a deletion along the short arm of chromosome 18. These statistics suggest that many patients undergo multiple deletions, which may indicate that the propensity for the development of the

disease is dependent upon sequential, recessive mutations, as was first generally hypothesized for cancer over two decades ago .[23]

Cytogenetic abnormalities are often seen in osteosarcoma cells. These cells tend to be highly aneuploid, with up to eight times the normal diploid chromosome number. A direct relationship has been demonstrated between the percentage of diploid tumor cells and the survival of the patient.[8]

Ionizing radiation is implicated in three percent of osteosarcoma cases; in these cases, cancers developed after an average interval of 12-16 years. Moreover, four percent of all patients with osteosarcoma have had radiotherapy for other tumors.[3]

Oncogenesis may also be related to growth factor abnormalities. Some osteosarcoma cells have been found to overproduce the cytokine PDGF (platelet-derived growth factor);[8] this is thought to result from high expression of the *c-sis* proto-oncogene.[24] Significant amplification of the c-*myc* proto-oncogene has also been evident in osteosarcoma tumor tissue. Osteosarcoma cells also tend to have an inappropriately high number of growth factor receptors,[8] which may contribute to the transformation process.

There are also several genetic diseases which tend to predispose patients to the development of osteosarcoma. For example, among patients with retinoblastoma, half of all secondary malignancies are osteosarcoma, and this type of cancer arises 2000 times more frequently in the skull and 500 times more frequently in the extremities than the rate for the general population.[8] Among patients with Paget's disease (a noncancerous disease involving bone deformity and increased osteoblastic activity), two percent develop osteosarcoma; multiple bone involvement is frequent, and these patients generally have a poor prognosis.[25] Finally, there is a significantly higher rate of osteosarcoma among patients with the congenital disease osteogenesis imperfecta.[2]

Conventional treatment. Osteosarcomas are generally difficult to manage. Standard treatment for osteosarcoma has traditionally involved amputation of the affected limb at the joint proximal to the affected bone. Until 1972, amputation was the only available treatment course, which had limited success and invariably changed the quality of these patients' lives.[26] In a thirty-year study (concluded in 1976), 1256 patients were treated with surgical ablation; 50% developed metastasis within six months, and 248 (19.7%) survived five years after diagnosis.[27] More recently, limb salvage procedures (including autologous grafts, allografts, and endoprothesis) have been introduced.[28] The

goals of this surgery are the complete removal of the tumor and avoidance of local recurrence while reconstructing a functional extremity. However, these types of procedures often carry an increased risk of recurrence without offering a significant benefit in terms of physical and psychological recovery.[29,30] For osteosarcomas not involving the extremities, reductive surgery is indicated, and the prognosis is generally poor.[2]

Osteosarcomas are generally radiation insensitive and relatively drug-resistant. However, studies in 1981 and 1982 showed a significant benefit of post-operative chemotherapy, which was aimed at eliminating micrometastases.[31,32] More recently, pre-operative chemotherapy has been used to shrink the tumor mass prior to surgery; this also seems to be a good prognosticator of overall success, as responders tend to have fewer recurrences. Also, the response to pre-operative chemotherapy allows a determination of the drug sensitivity of individual cancers, and allows the post-operative therapy to be designed accordingly.[33] Aggressive chemotherapeutic regimens show the greatest efficacy (as well as the greatest toxicity), and involve a multi-drug combination of any of the following: adriamycin, cisplatin, high-dose methotrexate, and ifosfamide.[34,35]

The treatment of metastases to the lung is generally disappointing, and usually involves thoracotomy to remove the portion of the lung which has undergone malignant transformation. Late-appearing (i.e., six months post-operative or later), single metastases have a better prognosis than do earlier or multiple metastases. However, the overall two-year survival of these patients is generally below 30%.[36] Patients with metastases to bony sites tend to do less well.[37]

The outlook for osteosarcoma patients is improving. However, the most recent available data shows that, with surgery and chemotherapy, 40% of all osteosarcoma patients experience relapse within five years. Secondary episodes seem to be more debilitating and less amenable to treatment, as only 30% of relapsing patients survive beyond five years.[3]

Gene Therapy

Gene therapy may be defined as a technique in which a functioning gene is inserted into the somatic cells of a patient to correct an inborn genetic error or to provide a new function to the cell. The use of genetic therapy to treat human disease first became conceptually feasible with the development of recombinant DNA technology, and has since progressed rapidly from speculation to reality.[38]

General Principles of Gene Delivery. Two basic approaches are possible with regard to gene delivery to tumor cells. First, cells can be taken out of the body, genetically-altered *ex vivo*, and then reimplanted. Classical means of gene transfer include a variety of techniques. Models for chemical transfer have included transfection by coprecipitation with calcium phosphate or dextran, and the use of polycations or polylipids to complex with the DNA of interest . In this system, the mixtures are introduced to a suspension of recipient cells; the chemicals disturb the plasma membrane to admit the DNA.[39] Physical techniques have included electroporation and microinjection. Each of these methods are nontargeted and relatively inefficient, as integration may occur in less than one percent of the cells.[40] More recently, *ex vivo* alteration has been done using retroviral vectors.

The second basic approach in clinical gene transfer is *in vivo* gene transfer. *In vivo* gene therapy has the advantage of circumventing tumor cell culture. In culture, cells from many tumors do not grow for a sufficient duration to permit genetic modification. In addition, this procedure is costly and labor intensive. One variation of this approach is to graft murine retroviral vector producer cells (VPC) into tumors. Highly effective gene transfer can be accomplished, as the VPC continuously produce vectors within the tumor. In xenogeneic models, murine VPC survive approximately two weeks and are thought to be able to actively produce retroviral vectors over that time period. Since only a portion of the tumor cells is undergoing mitosis at a given point in time, the constant exposure of cells to the retroviral vectors increases the likelihood of genetic integration. Accordingly, the efficiency of transduction is greater than that which can be accomplished with injections of retroviral supernatants.

The Clinical Inception of Gene Therapy. An *ex vivo* method of gene therapy employing retroviral delivery was utilized in the first trial of human gene therapy for adenosine deaminase (ADA) deficiency, a form of severe combined immunodeficiency,

which began in 1990.[41] In this protocol, T-lymphocytes were removed from two children with the disease, transduced *ex vivo* with retroviral vectors containing a normal copy of the adenosine deaminase gene, and then re-infused. Twenty-three intravenous infusions of genetically altered autologous T-cells have been administered to date to each child. Both children show evidence of improved immune function and neither has demonstrated any evidence of adverse effect due to the genetically-altered cells.[42,43]

The first gene marking experiment in humans also utilized *ex vivo* delivery by retroviral vectors. This study involved the treatment of 10 patients with autologous tumor infiltrating lymphocytes (TIL) that were transduced *ex vivo* with a retroviral vector encoding the neomycin resistance (NeoR) gene. None of these patients have demonstrated any untoward effects secondary to receiving the genetically-altered cells.[44]

The first cancer gene therapy protocol in 1991 was a direct extension of the NeoR/TIL gene marker protocol. Here, the gene for tumor-necrosis factor (TNF) was incorporated into the retroviral vector.[45] This gene is a powerful anticancer agent in experimental animals, but the induction of serious toxicity in patients receiving low levels of TNF underlined the need to study clinically relevant levels for this cytokine.[46] Since this time, the use of other immunostimulatory genes (such as IL2) in gene therapy protocols has been under intense investigation.[47]

Retroviral Vectors.[48,49] A retroviral vector is a genetically modified retrovirus in which a portion of the normal viral genome has been deleted and can be replaced with a new gene or genes to be delivered to target cells. In contrast to chemical and physical methods of cellular transfection, murine retroviral vectors have proven to be extremely efficient for gene transfer into mammalian cells. Efficiencies as high as 90% have been achieved in cultured murine fibroblast cell lines. Murine retroviral vectors differ from adenoviruses and herpes simplex viral vectors in that retroviruses will only integrate and subsequently express their genes in dividing cells. Because a defining characteristic of malignant tumors is rapid cell division, retroviruses are especially applicable in this situation.

The Moloney Murine Leukemia Virus. The Moloney murine leukemia virus (MoMLV) is a naturally occurring retrovirus which was serendipitously isolated from field mice;[50] its biological characteristics subsequently attracted great scientific interest. MoMLV contains a promoter within the 5' long terminal repeat (LTR) which regulates the transcription of viral RNA.[51] This RNA contains three retroviral gene regions (gag, pol,

and env) and the ψ packaging sequence.[52] The gag region encodes core viral proteins, the pol region encodes the reverse transcriptase and integrase genes, and the env gene region encodes viral coat proteins. Most retroviral vectors also contain an internal promotor (such as the SV40 early promoter) to drive transcription of the gene of interest. Retroviral vectors commonly include a selectable marker such as the bacterial NeoR gene, which encodes NPT II (neomycin phosphotransferase II), an enzyme that protects cells from the toxic effects of G418 (a neomycin analog).

Packaging and Producer Cell Lines. The ψ packaging sequence is required for the packaging of the viral RNA into an infectious virion. Cell lines have been engineered to delete the ψ sequence but retain the gag, pol and env genes. These cells, which produce "empty" virions, are termed retroviral packaging cells. The integration of genetically-altered MoMLV RNA containing the ψ sequence and the gene(s) of interest (along with alterations in the structural genes) into the cellular genome of packaging cells results in the production of infectious virions. These cells are called vector producer cells. These VPC encapsidate ψ-positive RNA which is packaged within the virions produced by translation of viral genes. The retroviruses thus produced are replication incompetent by virtue of the genetic deletion, and are only infective for a single generation.

The Infection Process. Retroviral infection of cells is a multistep process.[53] First, the retrovirus must bind to the target cell through a receptor on the cell membrane. The retrovirus is then internalized. Within the cytoplasm, reverse transcriptase is produced from the pol gene and converts the viral RNA genome into a double-stranded DNA molecule. Then, the integrase protein (also derived from the pol gene) inserts the retroviral genome into one of the cell's chromosomes. Integrase may recognize and bind a particular host DNA sequence, so the process may be specific rather than random.[54] Typically, only a single integration event occurs in a given cell.[54]

Safety. One safety concern with this system is that vector constructs containing inserted genes for use in gene transfer protocols could conceivably recombine with the gag, pol and env gene regions of MoMLV, thus generating replication competent wild type retrovirus. Because of this risk, the MoMLV vectors have been designed to minimize the possibility of recombination. Modification of the gag start codon to a stop codon and elimination of viral sequences needed in trans for the formation of the virus particle constitute the major modifications.[55] These precautions minimize the potential for the manufacture of replication-competent virus from producer cells which contain the vector.

Other Gene Delivery Methods. The further development of novel gene delivery methods is critical to the advancement of gene therapy for malignancies. Efficient gene delivery *ex vivo* into all tumor cells will likely be required for improved tumor immunization protocols. Likewise, improved gene transfer efficiencies will be critical for gene transfer into tumor cells *in vivo*. Currently, the inability to deliver genes to sufficient proportions of tumor cells or immune cells is a major impediment to the application of gene therapy to cancer.

Adenovirus. Adenovirus,[56,57,58] a nonintegrating virus, is one of the most promising gene delivery methods. Investigators have found that various portions of the viral genome can be deleted to improve safety. The initial adenoviral vectors have the E1A region deleted, which prevents viral replication. The gene of interest can then be cloned into the viral vector genome. Adenoviral vector genomes can accommodate up to about seven kb of foreign DNA and have the advantage of producing titers as high as 10^{12} pfu per ml. Adenoviruses are able to infect and express their genes in a wide host cell range with high gene transfer efficiency (over 90%) within the area of injection.

Adenoviruses enter the cell and disrupt the lysosomal membrane to escape destruction by the cell. The vector remains episomal while expressing its genes; integration into the host cell genome rarely occurs. Adenoviruses do not require cells to be actively dividing at the time of infection for gene expression. Because this virus infects a wide variety of tissues, gene transfer by this means is not selective for a specific tissue target but is specific to cells expressing the appropriate receptors. The use of tissue-specific promoters could theoretically limit gene expression to the target tissue, thereby mitigating the effect of transferred genes on normal tissues. These vectors have been developed primarily for the treatment of pulmonary disease secondary to cystic fibrosis[59].

Other Viral Vectors. Other viral delivery systems currently under development include adeno-associated virus (AAV)[60], herpes virus[61], and vaccinia virus[62]. Integration of AAV vectors into host DNA is reported to be somewhat less efficient than retroviral integration, but may be site-specific. Herpes viruses are naturally programmed for the persistence in nervous tissue, but the complex regulation of viral replication has made it difficult to produce stock vectors. The use of vaccinia virus and other RNA viruses for transient expression is also under investigation.

Nonviral Delivery. Nonviral delivery systems are also under active development. One nonviral method that has been used in clinical trials is DNA-liposome complexes.[63,64] Liposome technology increases the transfection efficiency of mammalian

cells by foreign DNA to higher levels than standard calcium phosphate procedures.[65] This method also has an advantage in that gene transfer can be accomplished *in vivo*. Receptor-mediated DNA delivery can be accomplished by linking DNA to a targeting molecule that binds to a specific cell surface receptor allowing internalization. Such a delivery method might allow for the delivery of larger DNA constructs. The gene transfer efficiency of these virus-free, nonintegrating methods is high. A variety of other DNA conjugation delivery systems are being pursued, but are now at preliminary stages of development.

Considerations in Gene Delivery. The choice of a gene delivery system for tumors is dependent on a number of factors, including the size of the gene, the necessity of integration, the desired length of time for gene expression, the level of expression required for a therapeutic effect, the nature of the host immune response to the delivery system, and the selectivity of the delivery method for the tumor tissue.[66] For example, to treat a neoplasm with a sensitivity gene such as the herpes simplex thymidine kinase gene, several principles come into play. First, since the object of the therapy is to destroy the tumor, permanent genetic alteration of the tissue is not an absolute requirement; once the tumor is gone, the gene is no longer needed. Alternatively, to cure a genetic disease, the gene would need to be integrated and expressed permanently in the deficient tissue for the life of the patient.

Second, expression of a sensitivity gene need only be at a sufficient level and duration to mediate tumor cell destruction. For this purpose, a nonintegrating vector could deliver a gene regulated by a cellular promotor that would maximize expression in the target tissue. As the tumor cells divide, a nonintegrated gene will be diluted since the episomal vector does not multiply within the cell. For actively dividing tumors treated with an episomal vector, repeated gene delivery may be required. The use of vectors that are selective for proliferating cells (e.g. retroviral vectors) or the use of promotors from genes expressed in a tissue-specific manner may enhance the effect of genes selectively transferred into tumor cells.

Suicide Gene Strategies.[67] Current suicide gene strategies aim to provide a cell with a new gene that is toxic itself or that provides an enzyme that can catalyze the conversion of a nontoxic prodrug to a toxic form. Thus, targeted cells receive a new enzymatic function that sensitizes the cell to a previously nontoxic drug. The antitumor effect is based on the selective gene transfer or expression within tumor cells. For

example, retroviral vectors which only will integrate into dividing cells allow for selective targeting of the enzyme to proliferating tumor cells over nonmitosising normal tissue in the same area. Another strategy that might be utilized is to selectively transfer the gene for a toxic product without the requirement of converting a prodrug to an active compound. If selective transduction of the gene can be done into tumor tissue, then the tumor can be preferentially destroyed. Likewise, gene expression of a toxic gene could be dependent on the activity of a tumor-specific promotor. Thus, even if gene delivery is not tumor-specific, the toxic gene would not be expressed in the absence of a tumor-specific promotor. These ideas represent a few examples as to how suicide genes could be utilized for anticancer therapy.

Herpes Simplex Thymidine Kinase/Ganciclovir System.[68,69,70,71,72] The HS-tk gene is a negative selectable marker or "suicide" gene. HS-tk sensitizes the transduced tumor cells to ganciclovir (GCV), an antiviral drug. GCV is a nucleoside analog which serves as a substrate for phosphorylation by HS-tk, resulting in a monophosphate (MP) form of the drug. Cellular phosphorylases convert this GCV-MP to GCV-triphosphate (GCV-TP) which inhibits primarily viral DNA-dependent-DNA-polymerase. The end result is the demise of the HS-tk transduced cells. Since the human thymidine kinase enzyme, which is normally present in human cells, has very low affinity for GCV, little systemic toxicity is observed.[73]

Cytosine Deaminase / 5-Fluorocytosine System.[74] Another negative selection marker has been adapted for use as a therapeutic gene. The bacterial gene cytosine deaminase (CD) is found in certain bacteria and fungi and normally deaminates cytosine to uracil. It was recently cloned from *E.coli* as a gene fragment that encodes a 42 amino acid open reading frame.[75] CD catalyzes the conversion of an innocuous compound, 5-fluorocytosine (5-FC) to a highly toxic compound, 5-fluorouracil (5-FU). The deamination reaction results in the generation of 5-FU which the cell then converts to 5-fluoro-2'-deoxyuridine 5' mono- and triphosphate. These metabolites inhibit both RNA and DNA synthesis and cell death becomes imminent.

The Bystander Effect.[76] A unique feature of the HS-tk/GCV anti-neoplastic activity is the observation that not all the tumor cells must contain the HS-tk gene in order to be killed by GCV. *In vivo*, the transplant to mice of a mixed population of tumor cells (consisting of as little as ten percent HS-tk positive cells and 90% HS-tk negative cells) has frequently resulted in the regression of the tumor mass upon treatment with GCV.[77]

The "bystander effect" also occurs *in vitro*. Various mixtures of HS-tk transduced tumor cells and nontransduced cells demonstrate complete inhibition of cellular proliferation by GCV when only one to 20% of the cell mixture was HS-tk positive. Since it is unlikely that 100% of the tumor cells in a patient's neoplasm could be successfully modified by genetic manipulation, this bystander effect may prove to be very important for successful antitumor responses and could afford gene therapy a greater flexibility as a treatment modality. Analysis of human melanoma, renal cell carcinoma, and neoplasms of breast, lung, and colorectal tissue reveals that all demonstrate the "bystander effect" *in vitro*.[78]

The mechanism of this "bystander effect" is not yet completely understood. The effect may involve the passage of toxic GCV-TP within HS-tk transduced tumor cells into adjacent HS-tk negative tumor cells, resulting in their death. This passage may be the result of fusion of apoptotic vesicles (derived of dying HS-tk-positive cells) with HS-tk-negative cells,[79] or it may be the result of metabolic cooperation, whereby phosphorylated GCV is passed from cell to cell via gap junctions.[80] Infected cells *in vivo* could potentially initiate an immune response that selectively targets tumor cells.[81] A soluble "bystander factor," secreted by infected cells and toxic to other tumor cells, has also been postulated. Such a factor could be a product encoded by infected cells or a product of GCV metabolism. Initial evidence supporting this soluble factor model is the finding that cell-to-cell contact is not necessary for bystander killing.[82]

The bystander effect does not seem to involve generalized, non-specific, cellular toxicity, since the overlying skin and other tissues surrounding these HS-tk treated tumors are not injured, while the tumors expressing the genes as well as the coincubated wild-type tumor cells are completely destroyed.[83] However, vascular endothelial cells which were recruited to nourish the tumor cells are destroyed, which may contribute to the efficacy of tumor regression.[84]

Ganciclovir

Ganciclovir (GCV) is a synthetic acyclic nucleoside analog of 2' deoxyguanosine, with a molecular weight of 255 daltons. It is a more potent derivative of acyclovir. GCV is the generic name for the chemical 9-(1,3-dihydroxy-2-propoxymethyl)guanine or DHPG. GCV is also known by the brand name Cytovene.[85] In therapeutic regimens, GCV exists in powdered form as a sodium salt (FW = 277.21). In this state, its chemical formula is $C_9H_{12}N_5 \cdot NaO_4$.

Utility. GCV is a clinically useful drug which is currently indicated for the management of cytomegalovirus (CMV) retinitis in immunocompromised patients (including AIDS patients) and for the prevention of CMV disease in transplant recipients. The origin of GCV dates back to 1982, when four labs simultaneously reported *in vitro* activity of the compound against CMV.[86] GCV has also been used experimentally against several other herpes viruses, including herpes simplex virus types 1 and 2 (HSV1 and HSV2), Epstein-Barr virus (EBV), and varicella-zoster virus (VZV).[87]

Mechanism. The mechanism of action by which GCV inhibits viral DNA synthesis has been studied extensively *in vitro* using human cells infected with CMV and HSV. After GCV has been transported into the cellular matrix, a viral thymidine kinase selectively phosphorylates the compound to GCV monophosphate; this is quickly converted to GCV triphosphate (GCV-TP) by cellular enzymes.[88] Direct incorporation of GCV-TP into viral DNA results in the termination of viral DNA elongation, as GCV-TP lacks the 3' hydroxyl group necessary for the addition of another nucleoside [89] A second mode by which GCV may inhibit viral DNA synthesis is by competitive inhibition of viral DNA polymerase.[90] GCV also inhibits cellular DNA polymerase α, but up to a 20-fold higher concentration is necessary,[91] indicating a preferential phosphorylation of GCV in virus-infected cells.

In vitro, GCV-TP is catabolized slowly, with 60 to 70% of the original level remaining in the infected cells 18 hours after removal of GCV from the extracellular medium.[92] Median effective HSV-inhibitory doses of GCV in several cell lines ranged from 0.2 to 3.0 μg/ml.[85] *In vivo*, GCV has a mean plasma half-life of 2.9 +/- 1.3 hours and is excreted renally.[85] A dosage range of 15-135 mg / kg / day for one to four weeks has been shown to be efficacious in the treatment of CMV in animal models.[93] At higher

levels, the clinical toxicity of GCV includes granulocytopenia and thrombocytopenia; in animal studies, GCV was carcinogenic, teratogenic and caused aspermatogenesis.[85]

Interleukin-2

Interleukin-2 (IL2) is a 15 kD glycoprotein secreted by activated T-cells that plays a central role in the generation of the immune response. IL2 has been called the "prototype cytokine,"[94] which reflects its pivotal functionality as well as its early discovery. The origins of IL2 date back to 1965 when a mitogenic activity was found in the conditioned medium of activated white blood cells.[95] Years later, it was noted that this activity maintained the growth of T-cells.[96] This discovery fostered the development of a quantitative microassay for the activity, which indicated that a single molecule was responsible.[97] This molecule is now recognized as IL2.

Since its inception (and prior to the implementation of a standardized system of nomenclature), IL2 has been known by several pseudonyms, including T-cell Growth Factor (TCGF), Thymocyte Stimulation Factor (TSF), Thymocyte Mitogenesis Factor (TMF), T-cell Replacing Factor (TRF), and Killer Helper Factor (KHP).[98] The variety of these early names, which were generally assigned on the basis of observed *in vitro* activity, is testament to IL2's wide-ranging activities.

Interleukin-2 Activities. The pleiotropic, receptor-mediated effects of interleukin-2 have been well-characterized. IL2 promotes the proliferation of T (and B) lymphocytes and stimulates other cytotoxic cell populations and macrophages. These activities will be expanded upon below.

Antigen-Specific Immunity. By stimulating the expansion of selected T- and B-cell clones, IL2 largely determines the magnitude and pace of the antigen-specific immune response.[99] IL2 is the primary regulator of the T-cell cycle, and is required for the progression from G_0 to G_1[100]. Moreover, progression of the T lymphocyte cell cycle has been shown to be solely dependent on three factors: IL2 concentration, IL2 receptor density, and the duration of the IL2 / IL2-receptor interaction.[101]

IL2 is also essential for the augmenting the generation of major T-cell subpopulations, notably CD4+ (helper) and CD8+ (cytotoxic) T-cells with increased cytolytic activity[102]. It has been shown that the mechanism behind this boosted cytolysis

is that IL2 augments the expression of the genes encoding the lytic molecules involved in cytolysis.[103]

Exposure to IL2 *in vitro* results in the production and maintenance of mature cytotoxic T-cells from terminally differentiated precursors.[104] This finding was later corroborated by the *in vitro* observation that IL2 can reconstitute a normal response from an acquired immunodeficient state.[105]

IL2 also participates in the promotion of B-cell function. IL2 makes possible the production of IgM pentamers by stimulating expression of J chain mRNA; it also potentiates the switch from the membrane form to the secretory form of μ heavy chains,[106] thus enhancing B-cell vigilance.

Antigen-Independent Immunity. IL2 is also an important stimulator of the nonspecific cellular immune response. IL2 causes the activation of phagocytic leukocytes, such as neutrophils and monocytes.[107] Exposure of macrophages to IL2 results in the induction of macrophage-mediated cytotoxicity,[108] as well as stimulation of antibody-dependent cell-mediated cytotoxicity.[109] Additionally, this cytokine participates in the tumoricidal activation of peritoneal macrophages.[110]

NK and LAK cells. Interleukin-2 also induces the proliferation and enhanced response of natural killer (NK) cells[111] and lymphokine activated killer (LAK) cells.[112] NK cells are large granular lymphocytes which participate in nonadaptive, non-MHC restricted cell-mediated cytotoxicity.[113] *In vitro* and *in vivo* evidence shows that NK cells are intimately involved in the destruction of tumor cells and the prevention of metastasis.[114] NK cells, which constitute about 3.6% of all lymphocytes,[115] are unique in that they express IL2-receptors constitutively and secrete IL2.[116] Thus, these cells are always immediately IL2-reactive and have the potential to be self-regulatory. A functional thymus is not required for the maturation of NK cells; rather, these cells are believed to originate and differentiate in the bone marrow.[117] The response of NK cells is of particular interest in that these cells constitute one of the few immunological weapons available in the limited arsenal of the athymic nude mouse. In fact, athymic rodents have been shown to have more pronounced NK activity than do their euthymic littermates.[118] LAK cells are non-MHC restricted cells which arise from peripheral blood lymphocytes after selection with IL2.[119] LAK cells are capable of lysing NK-resistant tumor cells, with minimal toxicity to normal cells.[120]

Secondary effects. The cascade-like secondary effects of IL2 are also of great importance. IL2 serves as an initiator molecule, which induces immune competent cells

to significantly alter their cytokine profiles. NK cells are stimulated by IL2 to secrete interferon-γ, GM-CSF, and TNF-α; activated T- and B-lymphocytes are stimulated to secrete IL-1ß, IL-4, IL-5, and IL-6.[98] These secondary cytokines are likely in turn to mediate potent immunologic and regulatory effects. For example, interferon induces expression of IL2 receptor alpha chain.[121]

Structure. The IL2 gene has been cloned and characterized at both the complimentary-DNA and genomic levels. Primary IL2 mRNA transcripts consist of four exons separated by one short and two longer introns.[122] The three-dimensional structure has been solved to a resolution of three angstroms.[123] The translated protein consists of 153 residues, with six alpha helices arranged in antiparallel fashion.[124] Elimination of the single disulfide bond greatly reduces IL2 activity, as do small changes in the primary or secondary structure.[125]

IL2 Receptor. Interaction of IL2 with its receptor (IL2R) is a necessary trigger for biological activity. The receptor consists of three distinct chains, each with about 215 amino acids in the external domain.[98] The alpha chain is 55 kD, anchored by 13 cytoplasmic residues.[126] The 75 kD beta chain has 286 cytoplasmic residues,[127] and the bashful gamma chain, only recently coaxed from hiding, is now known to be 64 kD with 86 internal residues.[128] These three chains, which constitute the IL2 binding site, cooperate kinetically to give a very high affinity ligand-receptor interaction (10^{-11} mol/L);[129] the affinity plummets when specific antibodies block any one of the three peptide chains.[125]

Clinical Immunotherapy. Use of IL2 as a therapeutic agent has been championed for years. Its role as an immune stimulant has been attractive in experimental treatment of immunodeficiency and infectious disease, and also as a vaccine adjuvant.[98] IL2 has been of particular interest in protocols for cancer immunotherapy. The goals of such therapy include the local release of low levels of IL2 capable of stimulating the immune system, activation of cytotoxic T-cells specifically directed against tumor cell antigens, and generation of immunological memory.[120]

In immune-competent mice, injection of tumor cells transduced with IL2 has resulted in the reduction of tumorigenicity and the development of protective immunity.[130] Thus far in human clinical trials, IL2 has been used to treat malignant

melanoma, breast carcinoma, renal cell carcinoma,[131] and acute leukemia.[132] To date, administration of this cytokine appears to have some efficacy in these settings: the combined response rate has been about 20% (with five percent experiencing a complete remission.)[98] However, results have somewhat tarnished by unpredictable responses and the onset of IL2 toxicity.

Toxicity. IL2 toxicity has been shown to be dose-dependent; the maximum-tolerated intravenous dose in humans is 10 mg as an immediate bolus, or 1 mg per hour over a 24 hour period.[133] At excess levels, toxicity is manifested in the form of fever, rigors, general malaise, myalgia, nausea, vomiting, vascular leakage syndrome, hypotension, and shock.[134] In some patients, IL2 has also been shown to produce a delayed-type hypersensitivity in the absence of antigen.[135]

Parameters of IL2 Administration. While toxic levels of IL2 have been defined, clinical agreement of safe yet biologically relevant levels remains more ambiguous. Clinicians generally report IL2 levels in terms of units, which is a derivative measure of functionality. One unit is defined as the "reciprocal of one final dilution of IL2 giving 50% of the activity of a standard IL2 preparation, determined by ^{3}H thymidine incorporation."[136] This definition implies that the unit is a relative measure that is not readily converted into a quantitative figure. However, a level of two units per ml has been reported to support growth of T-cells in culture[137].

In addition to the dose-dependent parameters, response to IL2 has been shown to be time-dependent. At minimum, a six-hour incubation with IL2 is needed to induce an increase in the proliferation of human peripheral blood mononuclear cells, and growth continues to increase with time.[101] The same source reports that an IL2 concentration of 100 picomolar (pM) is sufficient to saturate existing IL2 receptors, but a significant proliferative effect is seen with as little as 25 pM. It has been reported that 100 pM IL2 is sufficient *in vitro* to promote the proliferation of the ten percent subpopulation of NK cells that express high-affinity IL2 receptors; a 100-fold increase (10 nM) in IL2 concentration is necessary to stimulate the remaining 90% of NK cells, which have lower affinity receptors.[138]

The Athymic (Nude) Mouse

In 1968, it was reported that the nude mouse mutant (BALB/c, *nu/nu*) has a congenital absence of the thymus, and thus lacks T-lymphocytes.[139] This discovery suggested that this mouse strain was deficient in cell-mediated immunity. *Nude* is a mutant allele of the nude locus of the VII linkage group (chromosome 11).[140] The allelic deletion responsible for the loss of immunity also renders these mice hairless, which gives the term "nude" a double-entendre. Subsequently, it was reported that a human colon carcinoma grew as a xenograft when transplanted subcutaneously to nude mice.[141] Since then, the nude mouse has become an important model for studying human neoplasms and treatment modalities.

The rationale for employing the nude mouse model in cancer research is based on the supposition that transplanted human xenografts retain the characteristics of the original tumors. Comparative studies of original and transplanted tumors have shown that the chromosome pattern, DNA content, and histology of the tumor remains unchanged.[142,143] Moreover, the sensitivity of human tumor xenografts to therapeutic regimens is reported to correlate to experience of clinical treatment of the same tumor entity.[144]

Although the nude mice lack functionally mature T lymphocytes, they are not completely immunodeficient. These mice show a nearly normal response to T-cell-independent antigens.[145] B-cell growth and differentiation is not depressed in these animals, and thus high titers of natural antibodies may be present, which can react with tumor cells.[146] However, the lack of helper T cells would be expected to alter the antibody response to antigens. Secondly, tumoricidal macrophages can be isolated from nude mice,[147] which can contribute to the destruction and clearance of tumor cells. Perhaps most importantly, nude mice exhibit high levels of NK cell activity.[148] All three of these effector mechanisms contribute to the natural resistance of nude mice to transplanted tumors, and may account for some of the differences observed in the growth patterns of different tumor cell lines, as well as the individual variation seen in mice receiving identical implants.

Nude mice are especially susceptible to environmental contaminants and infectious disease. Under standard laboratory conditions, these animals fail to thrive and have an average survival time of only 14-30 days. However, this survival time can be increased to over two years by maintaining the mice under germ-free conditions.[149]

MATERIALS AND METHODS

Vector Construction

The retroviral constructs of all vectors used in these studies were derived from the Moloney Murine Leukemia Virus (MoMLV). The STK vector, obtained from F. Moolten (Veterans Memorial Hospital, Boston, MA) consists of the following construct: LTR--NeoR--SV40--TK--LTR.[150] This vector contains the herpes simplex type 1 thymidine kinase gene, driven internally by the simian virus-40 (SV40) early promotor. The vector is flanked by long terminal repeat (LTR) sequences; the 5' LTR is the promotor for the neomycin phosphotransferase II (NeoR) gene, which confers resistance to the neomycin analog, G418 (Gibco, Grand Island, NY).

The IL-2 vector (G1I2SvNa), obtained from Genetic Therapy, Inc.(Gaithersburg MD), has a construct of LTR-IL2-SV40-NeoR-LTR. The open-reading frame of the human interleukin-2 gene is located downstream of the 5' LTR sequence, and uses this sequence as its promotor. The SV40 early promotor serves as an internal promotor for the NeoR gene.

The BAG vector (G_1BgSvNa), also obtained from Genetic Therapy Inc., consists of the following construct: LTR-BAG-SV40-NeoR-LTR. BAG is the designation for an *Escherichia coli* ß-galactosidase gene which was excised from pBR322 plasmid and ligated into the retroviral backbone.

All three vectors are individually packaged by the amphotrophic retroviral vector producer cell line PA317, derived from NIH 3T3 fibroblasts (ATCC CRL# 6473). STK vpc are reported to generate a supernatant with a titer of 5-10 x 10^6 retroviral vector particles per ml. IL2 vpc are reported to generate a supernatant with a titer of 5-10 x 10^6 particles/ml. BAG vpc are reported to generate a supernatant with a relatively lower titer of 1-5 x 10^6 particles/ml. The titer of each vpc is pertinent to the transduction efficiency of target cells.

Cell Culture

Four human osteosarcoma cell lines (MNNG: CRL# 1547; MG63: CRL# 1427; TE85: CRL# 1543; G292: CRL# 1423) were obtained from American Type Culture Collection (Rockville, MD). A fifth osteosarcoma cell line (MLM) was derived at the Human Gene Therapy Research Institute (HGTRI; Des Moines, IA) from a lung

metastasis induced in an athymic mouse following orthotopic implantation to the tibial plateau of MNNG cells.

All cells were grown in complete media, consisting of 86% Dulbecco's Modified Eagle Medium (DMEM), with ten percent (by volume) heat-inactivated fetal bovine serum (The Salzman Corporation: Davenport, IA), and additionally 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone. (Except as noted, all of these tissue culture reagent were obtained from Quality Biological Inc.)

Cells were grown in Nunclon (Denmark) tissue culture flasks (175 cm^2 or 80 cm^2), and maintained in a Steri-cult 200 Incubator (Forma Scientific; Marietta OH) at 37°C, 95% humidity, and 5% CO_2. Tissue culturing was always performed under sterile conditions in a laminar flow hood (Forma Scientific).

Cells were passaged in the following manner: when confluent monolayers were achieved, the cells were washed in 10 ml Hank's balanced salt solution (HBSS; QBI) then exposed to trypsin-ethylenediamine-tetra-acetic acid (0.05%; QBI) at the minimal volume necessary to cover the bottom of the flask (4 ml for T-175 flasks and 2 ml for T-80 flasks). A five minute incubation at 37°C was sufficient to induce loss of cellular adherence. The cells were harvested by resuspending in complete media (10 ml), mixing thoroughly by agitation with a pipette and transferring to a sterile container (generally 50 ml Falcon centrifuge tubes). Washing the inside of the flask twice with HBSS (10 ml) completed the harvesting process. Harvested cells were counted with a hemocytometer, centrifuged at 1500 rpm for 5 minutes, and replated at dilutions ranging from 1:2 to 1:20, as needed. Medium was changed twice weekly, depending on the number of cells plated.

Cells used for injection into animals were resuspended in serum-free DMEM at the desired concentration, typically $2x10^7$ to 10^8 cells per ml. Cells not required for immediate use were frozen by resuspending in 70% complete media, 20% fetal bovine serum, and 10% dimethylsulfoxide (DMSO; ATCC) at a concentration of 10^7 cells per ml. The suspension was aliquotted into Corning cryovials (two ml size; Corning, NY) and frozen sequentially at -20°C, -70°C, and -150°C over a period of 48 hours.

Cell Counts

Enumeration of cells was performed in duplicate with a SPotlite hemocytometer. Two identical volumes (50 μl) of cell-containing media were transferred to each of two chambers of a 96 well plate into an equal volume of Trypan Blue solution (0.4%; Sigma: St. Louis. MO). A coverslip was placed on the hemocytometer, and the mixtures were

pipetted onto opposite sides. The volumes of original cell suspensions were diluted so as to have an estimated 200-400 cells per counting field. A total of eight fields were counted, and the results were averaged and adjusted according to the dilution factor to arrive at the total cell count.

Cellular Transduction with Retroviral Vectors

Human osteosarcoma cells (10^5) were plated in triplicate in a 6-well plate in 5 ml of complete media and allowed to attach overnight. The following morning, the supernatant (25 ml) from confluent T-175 cm^2 Nunc flasks of vector-producing cell lines (STK, IL2, or BAG) was removed. After the addition of protamine (5 μg/ml; Elkins-Sinn, Inc., Cherry Hill, NJ), the supernatant was passed through a 10 ml syringe (Becton-Dickinson) fitted with a 0.22 micron filter (PCG Scientifics, Gaithersburg, MD). Protamine, a polycation, is added to alter the cell surface charge to improve vector: cell-receptor binding. The filtered supernatant/protamine was then applied to the target cells (5 ml per well.) This process was performed six times at intervals of approximately 12 hours. The morning following the sixth transfer, supernatants were aspirated from the osteosarcoma cells and replaced with complete medium containing the antibiotic G418 (neomycin) at 1 mg/ml (active drug concentration: Gibco). Cells which had been successfully transduced with the NeoR gene were able to survive the ten to 14 day selection process. G418 resulted in the destruction of a substantial proportion of the tumor cells, suggesting a gene transfer efficiency of less than 10%.

Cellular Proliferation Assay

Cells at passage five to 15 were harvested and counted. One million cells were placed in a T-75 flask, and the time was noted. The cells were examined daily; when 90% confluency was achieved (usually 72-96 hours), cells were harvested and counted quantitatively in duplicate. Generation time was calculated according to the standard equation for logarithmic growth: $t_{gen} = 0.301\ t/ (\log X_t - 6)$, where t_{gen} = generation time, t = incubation time, X_t = final cell count, and $6 = \log_{10}(10^6)$.

In Vitro Bystander Assay

Four different mixtures of vector and target cells were made. The first involved STK-transduced cells as the vector and wild type cells as the target. The second involved a combination of equal amounts of STK- and IL2-transduced cells as the vector, and wild

type cells as the target. The third group involved IL2-transduced cells as the vector and wild type cells as the target, and the fourth used STK-transduced cells as the vector and IL2-transduced cells as the target. For each of the four groups, a total of eight mixtures were plated in triplicate in 96-well plates: 100% vector cells, 50% vector and 50% target, 25% vector and 75% target, 10% vector and 90% target, 5% vector and 95% target, 2% vector and 98% target, 1% vector and 99% target, and 100% target cells. See Table 1 below.

Table 1 : 96-Well Plate Formatted for Bystander Assay

	STK : NV	STK/IL2 : NV	IL2 : NV	STK : IL2
100 %				
50 %				
25 %				
10 %				
5 %				
2 %				
1 %				
0 %				

Wild type, STK-transduced, and IL-2 transduced cells were harvested, and diluted in complete media to a concentration of $5x10^4$ cells per ml. Appropriate mixtures of cells were made in triplicate by volume. Each well of the plate had a final volume of 0.2 ml, with a total of 10^4 cells per well in 96-well flat bottom plates, once for each cell line.. The plates were incubated at 37°C overnight to allow attachment of cells. The next morning, GCV (Ganciclovir; Syntex Laboratories Inc., Palo Alto, CA) was added as 5 μg/ml in HBSS, and the plate was incubated for 48 hours. Four hours prior to harvest, 5 μCu/ml tritiated thymidine (methyl-^{3}H, DuPont / Nen) was added to each well (50 μl of 20 μCu/ml stock). During this incubation, actively dividing cells incorporated the radiolabel into replicated DNA strands. After this time, media from each well was aspirated and disposed according to Radiation Safety Guidelines. Next, each well was rinsed once in HBSS (100 μl) to remove dead cells and unincorp□□ated label. Next, 0.05% trypsin (50 μl) was added, and after an incubation period of ten minutes at 37°C, the cells

were collected onto a filter strip by a cell harvester (Tomtec Inc., Orange, CT). Radioactivity was measured by a Beta Plate Reader (LKB Wallac, Gaithersburg, MD), and is expressed as the mean DPM (disintegrations per minute) of triplicate wells. Comparisons were made between radioactivity of target cells alone versus radioactivity with each coincubation percentage of transduced cells.

This assay was repeated for the MNNG line using a more rigorous experimental design with twelve replicates per treatment group to confirm the earlier results. The percentages of transduced cells were designed as a geometric progression (i.e., 1, 2, 4, 8, 16, 32, 64) to readily afford the evaluation of HS-tk / IL2 synergy. Control wells for each cell line without GCV and for cell-free complete media were included.

GCV Sensitivity Assay

Each cell type (wild type, STK-transduced and IL2-transduced) from a given osteosarcoma was harvest and diluted to $5X10^4$ cells/ml media as described above. Each cell type was plated in 12 wells of a 96 well plate (10^4 cells in 0.2 ml). After incubating overnight, GCV was added in 20 μl HBSS: three wells of each cell type received 2 μg/ml, three wells received 5 μg/ml, and three received 10 μg/ml as the final concentration. The remaining three wells of each cell type received 20μl of HBSS and no GCV. After a 44-hour incubation, the cells were radiolabeled, harvested, and measured for radioactivity as above.

X-Gal Staining

To evaluate the *in vitro* and *in vivo* transduction efficiency in cells of the BAG gene, ß-galactosidase expression was detected using an X-Gal histochemical stain.[151,152] Cells which had been passaged at least five times and which putatively contained the BAG gene due to G418 resistance (i.e., all five BAG transduced osteosarcoma lines and BAG vpc) were harvested and plated ($1x10^4$ cells per well in 2 ml media) in triplicate in six-well plates (Corning). The plates were incubated overnight at 37°C. The next day, the media was removed and replaced with a fixative solution (0.05% glutaraldehyde, 2 ml: QBI). The cells were fixed at room temperature for 15 minutes. Next, the fixative was aspirated to an appropriate container, and the cells were rinsed three times in HBSS. The second rinse was applied for about ten minutes to minimize the likelihood of leaving residual fixative. Next, X-gal solution (40 ml) was made by adding X-gal [5-bromo-4-chloro-3-indoyl-ß-D-galactosidase, 40 mg in DMSO (1 ml)] to the Reaction Mixture [39

ml of 5.0 mM $K_3Fe(CN_6)$ = potassium ferricyanide, 5.0 mM $K_4FE(CN_6)\cdot 3H_2O$ = potassium ferrocyanide, and 2.0 mM $MgCl_2$ in PBS]. Because X-Gal is photosensitive, care was taken to avoid prolonged or direct exposure to light. Sufficient solution to cover the cells (1 ml) was then added. The plates were incubated overnight at 37°C.

Staining with X-Gal turns ß-galactosidase-expressing cells blue when an indolyl is liberated from X-Gal by the action of ß-galactosidase, and subsequent oxidation and self-coupling forms an indigo blue derivative. A gradation from colorless to deep blue was seen, indicating a range in cellular expression. The vector-containing cells can thus be discriminated from unmodified cells and enumerated with light microscopy. Cells were scored as positive (strongly or moderately blue), and negative (no color change), and at least 500 cells of each line were visualized and scored. Control cells (each of the five wild type osteosarcomas as well as STK and IL2 vpc) were also collected and stained. All cells were negative for expression of the BAG gene.

ELISA Assay

An IL2 Immunoassay Kit was obtained from Pharmacia Biotech Inc. (Piscatawa, NJ). The kit consisted of an IL2 Microtiter Plate with 96 removable polystyrene microtiter wells coated with a murine monoclonal antibody against IL2, IL2 standard (10 ng), Assay Diluent RD1A (11 ml), Calibrator Diluents RD5 and DRD6E (21 ml each), Wash Buffer Concentrate (21 ml of 25x strength), Color Reagents A and B (12.5 ml each of hydrogen peroxide and chromogen (tetramethylbenzidine), and Stop Solution (6 ml of 2N H_2SO_4).

To perform the ELISA, Assay Diluent RD1A (100μl) was added to each well to be used, followed by cellular supernatants and standard solutions (100μl). Supernatants were collected and centrifuged immediately before use. IL2 standards were prepared by making six serial doubling dilutions from a 2000 pg/ml stock. All samples and standards were assayed in duplicate when possible. The microtiter plate was mixed gently and incubated at room temperature for two hours. Next, each well was vacuum aspirated and washed three times with Wash Buffer (400μl). IL2 conjugate (200μl) was added, and the plate was again incubated for two hours at room temperature. The aspiration/wash steps were then performed. Next, Substrate Solution (200μl) was added to each well. After a 20 minute room-temperature incubation, Stop Solution (50μl) was added to each well, and the color change was measured using a spectrophotometer (Hewlett-Packard) set at 450 nm. Data are expressed as picograms of IL2 produced per 10^5 cells per day.

Nude Mice

Congenitally athymic female nude mice (BALB/c, *nu/nu*) were obtained from Harlan-Sprague Dawley and housed in laminar flow cabinets in the nude mouse room at HGTRI. All mice were afforded autoclaved chow and water ad libitum throughout their stay and were kept for at least one week before any experimental manipulation. Three to six mice were housed per cage, and the mice were monitored daily by investigators and animal facility staff.

Injections. Mice were restrained by grasping the scruff of the neck between the thumb and index finger, then holding the tail with the fourth finger of the same hand. All injections were done with one, five, or ten ml syringes fitted with 27 gauge hypodermic needles (Becton-Dickinson). Injections of cells, diluted to the appropriate concentration in a volume of 0.05 to 0.2 ml HBSS, were done subcutaneously halfway between the diaphragm and pubic symphysis, using at least a 0.5 cm injection tract. The formation of a bleb was the criterion for a successful injection. Intratibial injection was done after effecting anesthesia by momentarily placing the nose of the mouse in a 50 ml conical tube containing a gauze soaked in Metofane (methoxyfluorane: Pitman-Moore, Inc., Washington Crossing, NJ). The shaft of the bone was penetrated through the tibial tuberosity 1 cm into the shaft by using the needle like a drill.

GCV Treatment. GCV (3 mg in 1 ml HBSS) was injected intraperitoneally; GCV injections were done twice daily at ten to 14 hour intervals for a period of 12 days. Mice experiencing toxic effects were given reduced doses (2.5 mg/ml) for the remainder of the treatment period.

Assessment. Mice were evaluated daily for tumor size using calipers. Mice with tumors exceeding 20 mm by 20 mm, as well as mice having completed follow-up evaluation, were euthanized with CO_2 or by cervical dislocation.

Preparation of Tissue for Histology

Tissue samples were obtained by sterile dissection, then placed in a solution of 5% formaldehyde. Specimens were then stored at room temperature. Tissue sectioning and hemotoxlyin and eosin staining were done contractually off-site.

Tumor Harvest

After euthanasia, each tumor was isolated in sterile fashion in a laminar flow hood using a scalpel, then minced to fine pieces in a petri dish, suspended in 15 ml complete media, and transferred to a 50 ml conical centrifuge tube. Filtered tumor digestion media (15 ml, consisting of collagenase (1 mg/ml), DNase (0.1 mg/ml), and hyaluronidase (15 units/ml) in HBSS: all from Sigma) was added. The tube was then agitated horizontally at a moderate speed for 30 minutes on a Red Rotor shaker (Hoefer Scientific Incorporated, San Francisco CA), then allowed to stand vertically for two minutes to let large particles settle. The liquid (excluding settled particles) was then transferred to a clean tube and centrifuged at 1500 rpm for five minutes. Pelleted cells were resuspended in media, counted, and cultured in the standard fashion.

Statistical Analysis

Significance of results was calculated at 95% confidence limits using Student's t distribution, according to the equation $t = (X - \mu) / (s / (n)^{0.5})$ with n-1 degrees of freedom, where t = confidence limits, X = mean experimental value, μ = interval of significance, s = standard deviation, and n = number of replicates. Microsoft Excel was used as a tool to determine standard deviation and mean values

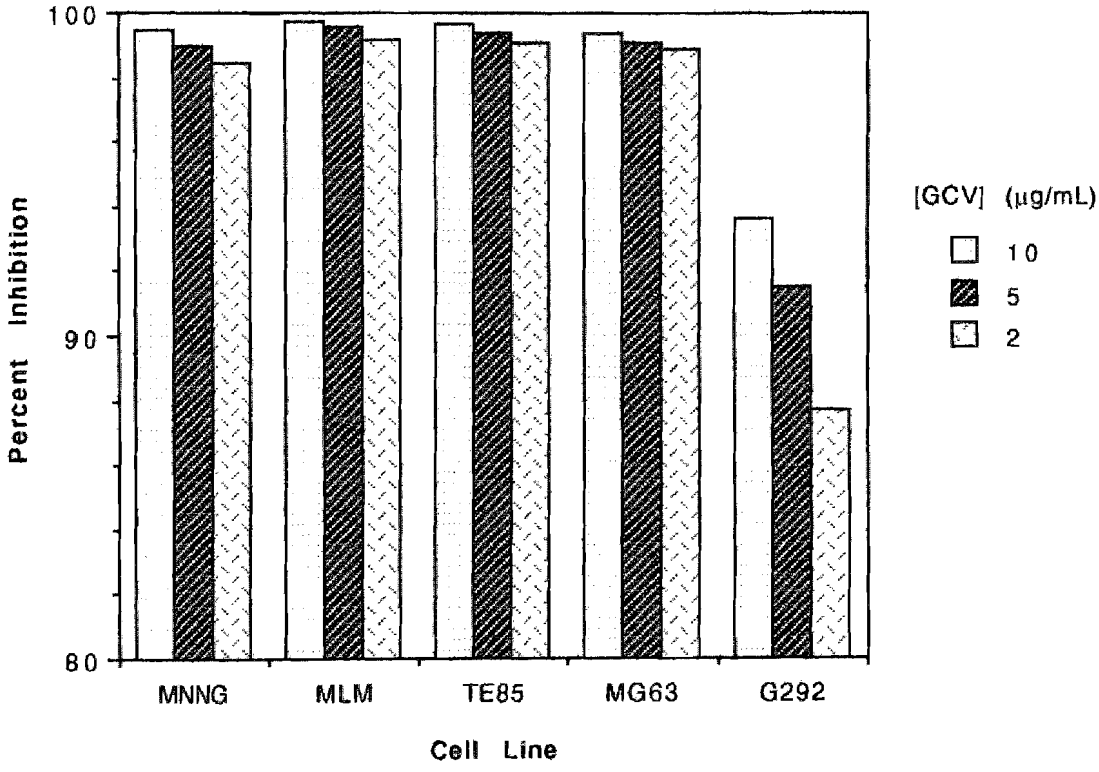
RESULTS

In Vitro

Five human osteosarcoma cell lines (MNNG, MLM, TE85, MG63, and G292) were studied, and each of these lines was stably and separately transduced (via retroviral vectors) with three recombinant genes: Herpes simplex type 1 thymidine kinase (STK), human interleukin 2 (IL2), and bacterial ß-galactosidase (BAG). Throughout this text, the cell lines thus generated are designated by the parental abbreviation followed parenthetically by the vector. The designation (NV) indicates the "no-vector" wild type tumor cell line. Expression of gene products from all cell lines was determined empirically. Each cell type was studied as to proliferation rate, GCV sensitivity, and the potential for bystander effect. Throughout this text, the term "proliferation" is used interchangeably with "thymidine-incorporation" for the sake of simplicity; in the strictest sense, the extent of thymidine incorporation is only directly proportional to the level of proliferation, in that cells which incorporate the radiolabel are both the original, parental cells and the products of multiple division cycles.

Assays for transduction of functional vectors. A functional assay for GCV sensitivity was performed on all STK-transduced lines surviving G418 selection (fig. 1). The GCV-mediated inhibition was dose-dependent, and had only a small inhibitory effect on control (NV) cells. Four of the five cell lines showed high susceptibility to low levels of GCV; in each case, at least 98% decrease in proliferation (as compared with control cells of the same type receiving no GCV) was seen at 2 μg/ml GCV. At higher levels of GCV (10 μg/ml), the percent decrease in proliferation ranged from 99.4 to 99.8%. The fifth line (G292) showed less sensitivity compared to the other four lines, with 83.7% inhibition at 2 μg/ml, up to 93.7% at 10 μg/ml. The wild type (NV) and IL2-transduced lines, tested along with the STK-transduced lines, showed slight susceptibility to GCV (addressed further below).

FIGURE 1: GCV Sensitivity of Five Osteosarcoma Cell Lines Transduced with the STK Vector



An IL2 ELISA was performed on supernants from confluent flasks of all IL2 transduced lines. All five lines, as well as IL2 vector producer cells, tested positive for production of antibody-recognizable IL2 (fig. 2). Two IL2-transduced lines (MNNG and MLM) showed very high production (74.38 and 57.16 pg / 10^5 cells / day respectively). Three lines (IL2 vpc, MG63, and TE85) showed moderately high levels of IL2 production (31.32, 24.06, 20.44 pg / 10^5 cells / day respectively). The other cell line, G292, showed relatively low IL2 production (8.23 pg / 10^5 cells / day). All non- IL2-transduced lines (including STK and BAG vpc) were also tested; the absorbance readings for these lines were not significantly different ($p > 0.05$) from the background level (i.e., less than 0.315 pg / 10^5 cells / day).

X-Gal staining was performed on all BAG-transduced lines and the BAG vpc (fig. 3). Four of the transduced osteosarcoma lines showed a strong majority of positively stained cells (MNNG: 79.1%; MLM: 85.4%; TE85: 76.8%; MG63: 89.8%), closely approaching the percent positive BAG vpc (91.1%). For the G292 (BAG) line, less than half (48.6%) of cells stained positively. Non-transduced lines and STK and IL2 vpc were completely negative.

FIGURE 2: **ELISA-Confirmed Human-Specific IL2 Production by Five IL2-Transduced Osteosarcoma Cell Lines and Murine IL2 Vpc**

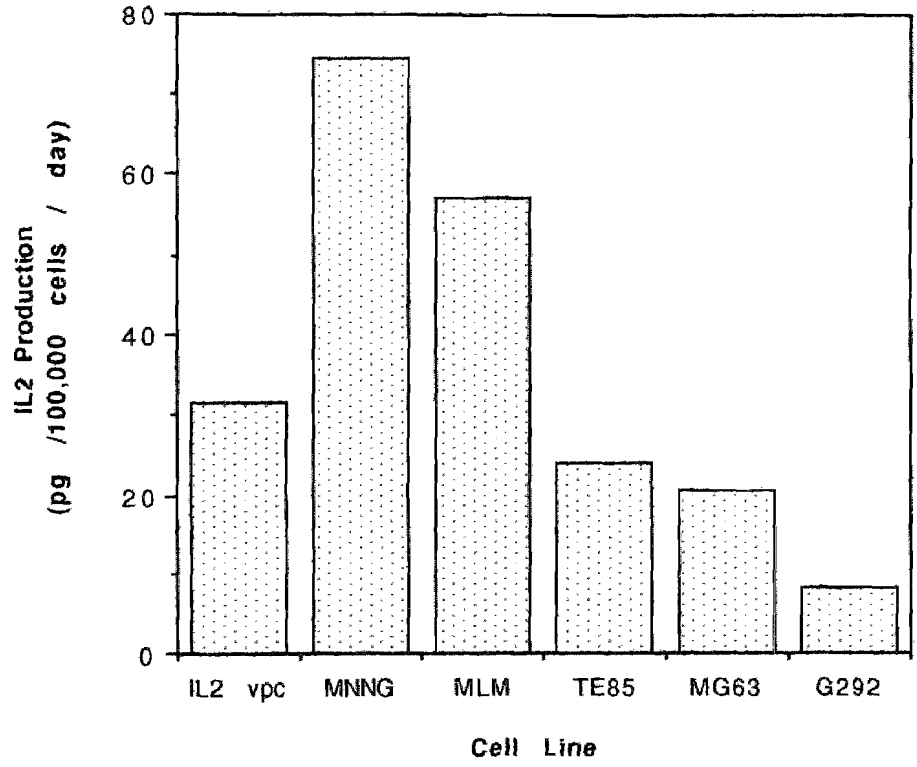
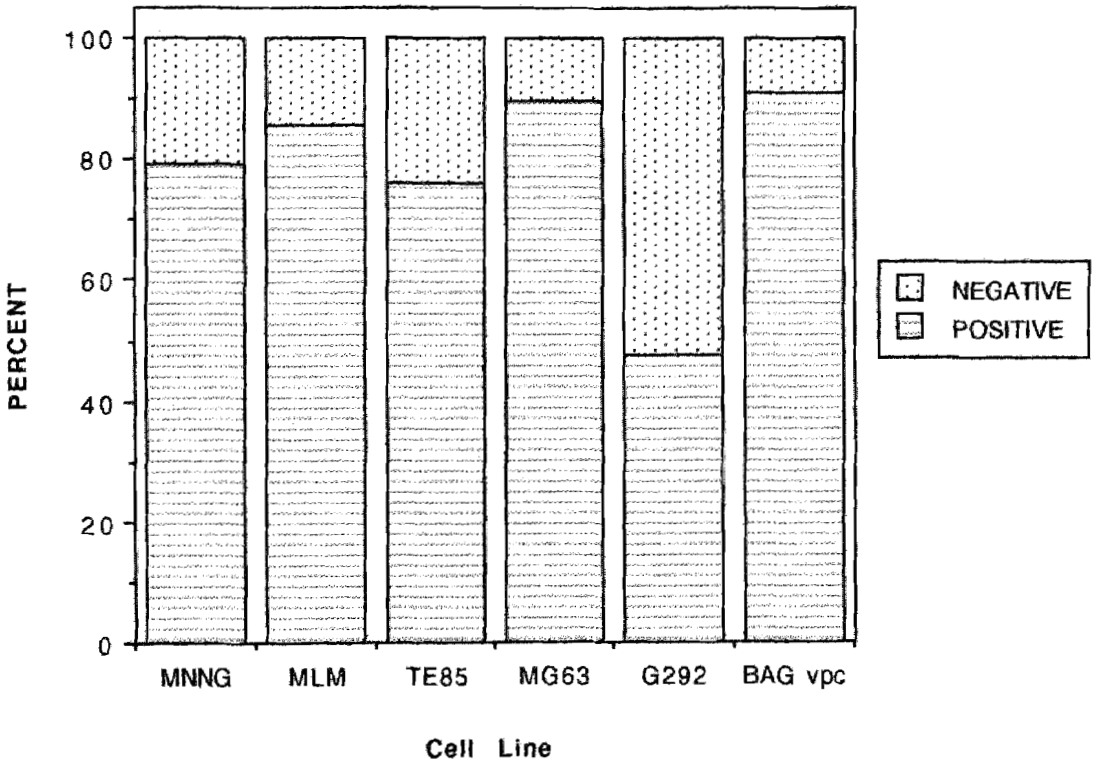


FIGURE 3: ß-Galactosidase Expression as Determined by X-Gal Staining of BAG-Transduced, G418-Selected Osteosarcoma Cells and Murine BAG Vpc



Cellular Proliferation Assay. Table 1 shows the experimentally determined generation times for each of the 23 cell lines used in this project.

TABLE 2: Growth Rates for Twenty Osteosarcoma Lines and Three Vector-Producer Cell Lines

CELL LINE	GEN. TIME (HOURS)	CELL LINE	GEN. TIME (HOURS)
MNNG (NV)	24.9	MG63 (NV)	39.1
MNNG (STK)	26.9	MG63 (STK)	50.7
MNNG (IL2)	26.9	MG63 (IL2)	43.1
MNNG (BAG)	28.5	MG63 (BAG)	23.2
MLM (NV)	27.0	G292 (NV)	81.0
MLM (STK)	23.2	G292 (STK)	81.6
MLM (IL2)	31.4	G292 (IL2)	86.7
MLM (BAG)	23.0	G292 (BAG)	51.9
TE85 (NV)	24.7	STK VPC	26.4
TE85 (STK)	20.3	IL2 VPC	24.3
TE85 (IL2)	23.4	BAG VPC	19.4
TE85 (BAG)	24.6		

Doubling times ranged from a minimum of 19.4 hours (BAG vpc) to a maximum of 86.7 hours (G292 BAG). Three of the five osteosarcoma lines showed a narrow range of variation in growth time. Generation times for the MNNG line showed a 12.5% range, from 24.9 hours (NV) to 28.5 hours (BAG). The MLM line showed a 26.8% variation (23.0 hours for the BAG-transduced line, to 31.4 hours for the IL2-transduced line.) Generation times for the TE85 line ranged from 20.3 hours (STK) to 24.7 hours (NV), or 17.9%. The other two osteosarcoma lines showed greater variation. A 54.3% variation was seen between the different MG63 lines (23.2 hours for the BAG-transduced line to 50.7 hours for the STK-transduced line). A range of 40.1% was observed for the G292

line (51.9 hours for the BAG-transduced line to 86.7 hours for the IL2-transduced line. The G292 cells showed a significantly ($p < 0.05$) slower doubling rate compared to the other cells lines.

Retroviral-mediated transduction with each of the three genes (STK, IL2, and BAG) did exert an effect on the generation time of each of the five human osteosarcoma tumor cell lines; however, the effect was not uniform for each gene. STK-transduction resulted in a significantly slower generation time in two of the five lines (MG63: 29.5%; MNNG: 7.8%). In two lines, transduction with the STK gene resulted in cells with a faster generation time (TE85: 17.9%; MLM: 14.3%). IL2-transduction resulted in slower growth in four lines (MLM: 15.9%; MG63: 10.2%; MNNG: 7.8%; and G292: 7.1%). BAG-transduction resulted in faster growth in three lines (MG63: 40.8%; G292: 35.9%; MLM: 15.2%). In the MNNG line, BAG transduction resulted in a 12.5% slower generation times. For cell lines not noted above, transduction resulted in no significant diference in generation time.

Generation times were also determined for the three vector producer cell lines. While they were comparable, the BAG vpc replicated the most rapidly of these lines, and was the fastest growing cell line overall, with a doubling time of 19.4 hours. The IL2 vpc had a generation time of 24.3 hours, and the STK vpc doubled every 26.4 hours.

GCV Sensitivity Assay. (Fig 1). Each cell type within each osteosarcoma line was incubated in three different GCV concentrations (0, 2, 5, and 10 μg/ml) for 48 hours. Only cells insensitive to GCV are expected to survive this challenge. Four hours prior to harvest, the cells were exposed to tritiated thymidine. Actively dividing cells incorporate the radiolabel during their replicative processes. Thus, the level of radioactivity (measured as disintegrations per minute or DPM) is proportional to the number of cells surviving incubation with GCV. The DPM for the GCV-negative wells were used as the control for comparison within each cell type. In four of the five osteosarcoma lines, GCV-mediated inhibition at 10 μg/ml exceeded 99.4% for the STK-transduced lines. Thus, susceptibility to GCV was acquired by the transduced cells, confirming that these tumor cells express functional HS-tk. Wild-type lines did show some sensitivity to GCV with a decrease in growth rate ranging from 35.9% (G292) to 68.4% (MLM) at the highest concentration of GCV.

In two of the five osteosarcoma lines (MLM, and G292), transduction with IL2 induced a protective effect; i.e., these cells showed an increased level of survival as

compared with the corresponding NV lines. In the other three lines, susceptibility of IL2-transduced cells to GCV was not significantly different to that seen in the non-transduced cells. Table 2 below shows the percent inhibition (compared with control cells receiving no GCV) for each cell line at the highest concentration of GCV tested. The complete results of these assays (expressed in DPM for each cell line at all tested levels of GCV) are depicted below (figs 4-8).

FIGURE 4: In Vitro GCV Sensitivity Assay - MNNG Human Osteosarcoma

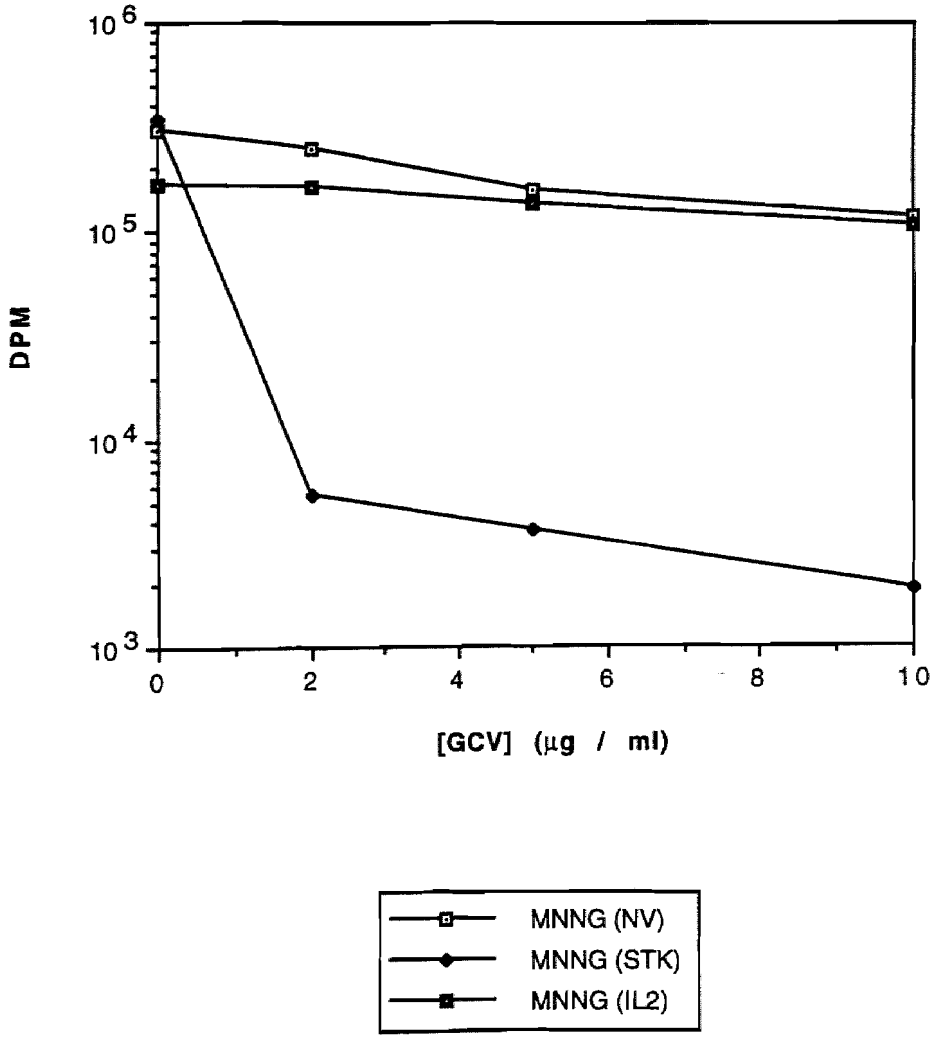


FIGURE 5: In Vitro GCV Sensitivity Assay - MLM Metastatic Osteosarcoma

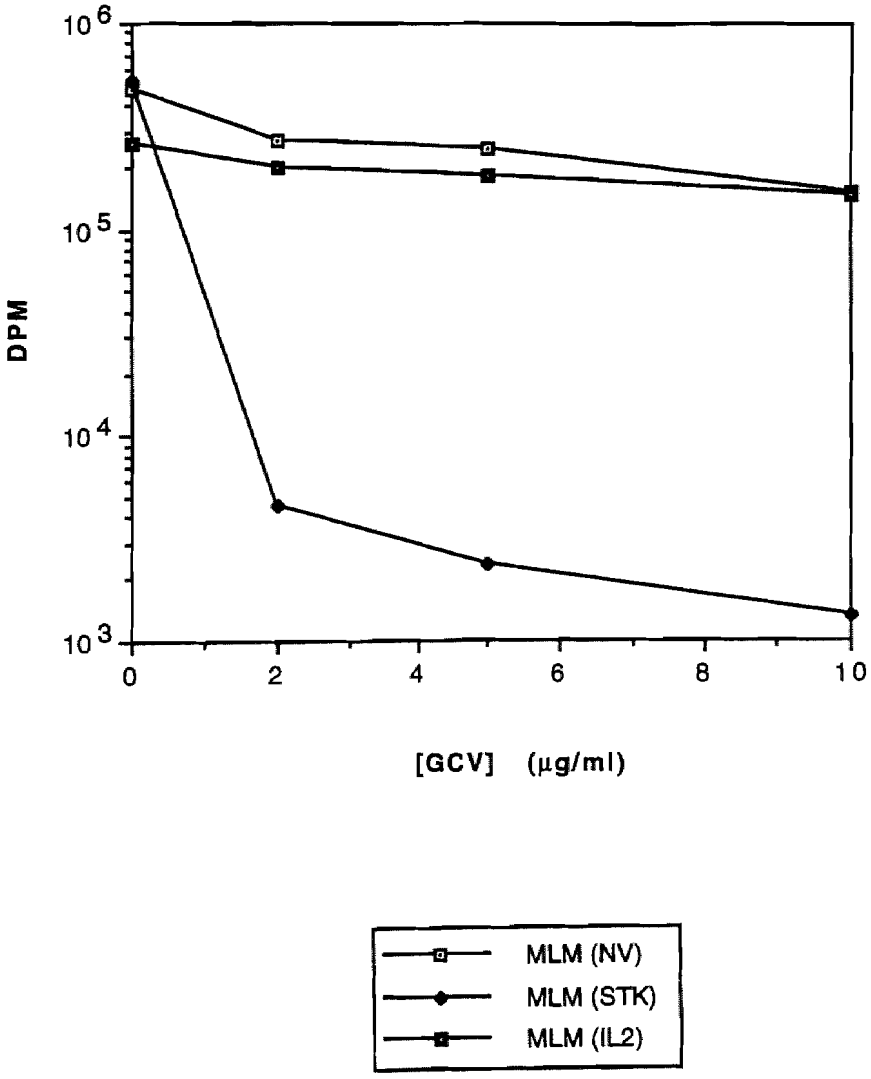


FIGURE 6: In Vitro GCV Sensitivity Assay - TE85 Human Osteosarcoma

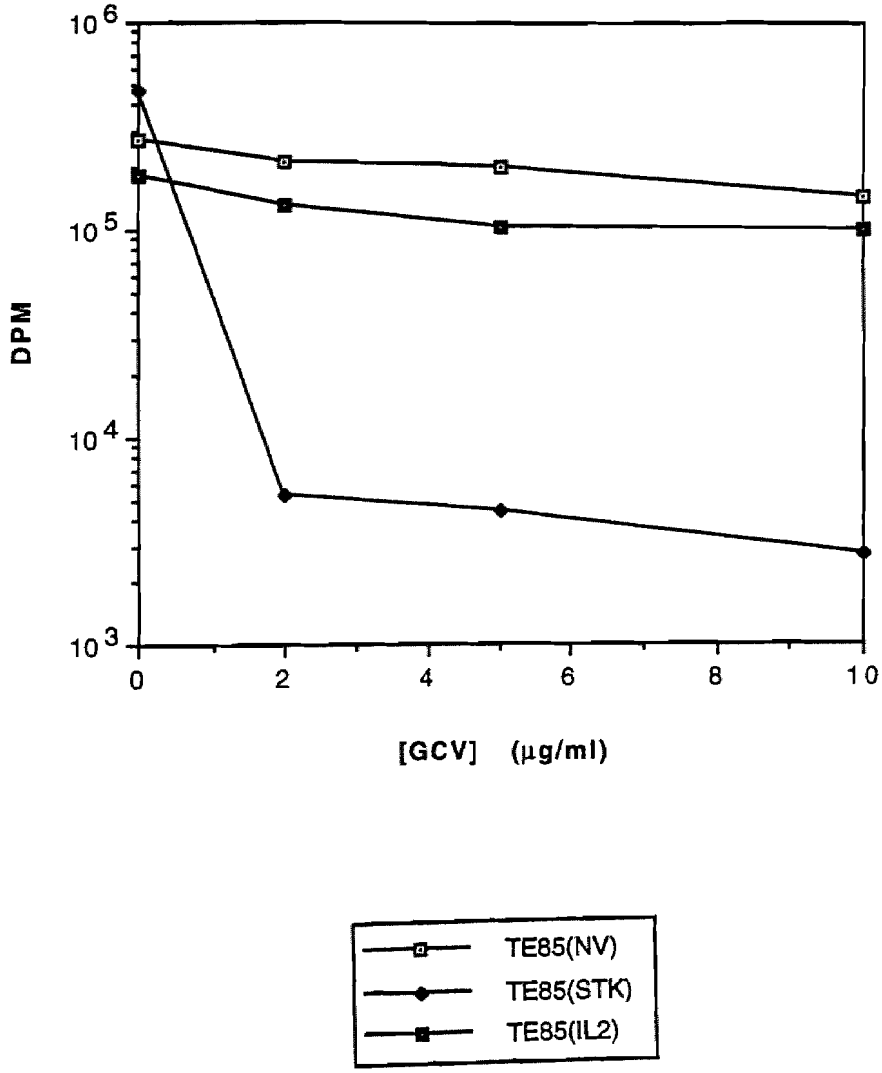


FIGURE 7: In Vitro GCV Sensitivity Assay - MG63 Human Osteosarcoma

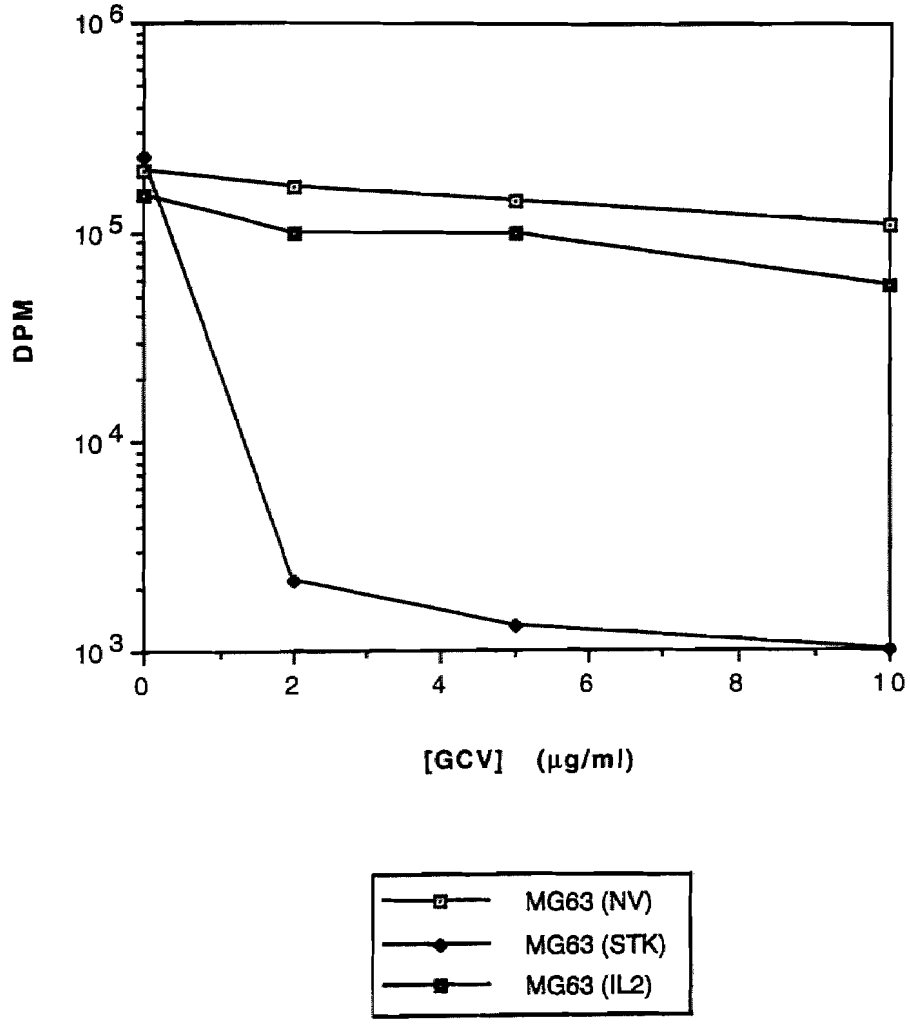


FIGURE 8: In Vitro GCV Sensitivity Assay - G292 Human Osteosarcoma

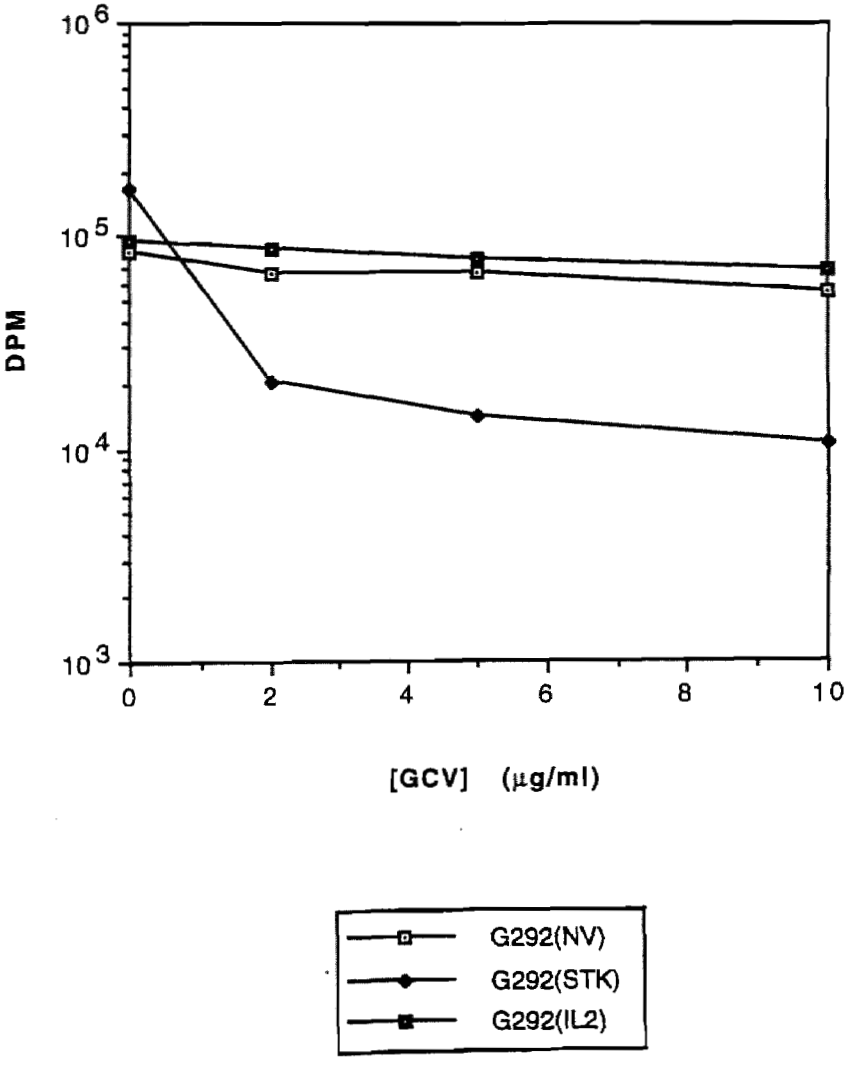


Table 3: Percent Inhibition at 10 μg/ml GCV

Cell Line **Vector**	MNNG	MLM	TE85	MG63	G292
NV	62.0	68.4	46.6	45.9	35.3
STK	99.5	99.8	99.4	99.7	93.7
IL2	36.5	44.2	44.2	63.4	27.1

The GCV sensitivity of MNNG was examined more carefully using twelve replicates of each cell type incubated with no GCV and in 5 μg/ml GCV. In each case, the results are statistically significant at the 95% confidence interval ($p < 0.05$). The MNNG (STK) showed a 99.3% decrease in proliferation (1569 +/- 254 DPM, versus 219,321 +/- 28,021 DPM) when incubated in 5 μg/ml GCV as compared to GCV-free control MNNG cells. The wild type MNNG line showed a 50.1% inhibition (79,941 +/- 11,584 DPM versus 160,283 +/- 31,256 DPM) in 5 μg/ml GCV. The MNNG (IL2) line was also affected by incubation in GCV; a 52.0% decrease (84,856 +/- 16,130 versus 176,796 +/- 37,647 DPM) was observed in the presence of 5 μg/ml GCV. The differences between the IL2-transduced and wild type MNNG cells are not statistically significant ($p > 0.05$).

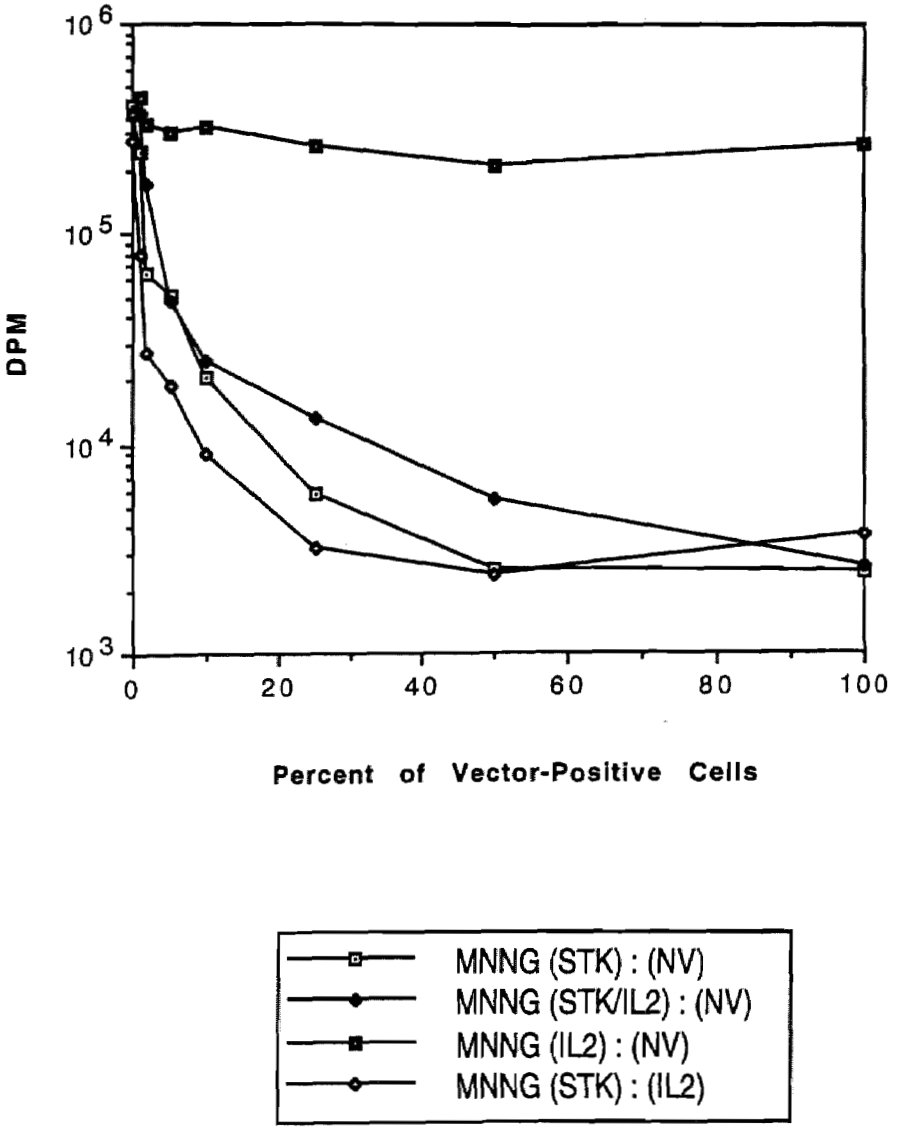
In Vitro Bystander Assay. This tritium incorporation assay was performed on each of the five cell lines to determine the ability of transduced cells to cause the concurrent inhibition of unmodified cells. Individual results are described below.

MNNG Coincubation of MNNG (STK) and MNNG (NV) cells resulted in a significant inhibition of growth at each percent STK tested. An equal mixture of MNNG (STK) and MNNG (NV) cells resulted in proliferation which was 99.4% decreased from the control of 100% MNNG (NV) cells. With a mixture containing 10% MNNG (STK) and 90% MNNG (NV), a 94.9% decrease in proliferation was seen. At 2% MNNG (STK), a 84.25% inhibition was seen. When MNNG (NV) cells were coincubated with both MNNG (STK) and MNNG (IL2) cells, a similar trend is seen, though proliferation is decreased less at a given concentration of MNNG (NV) cells. For example, at a 50:25:25 mixture of MNNG (NV), MNNG (STK), and MNNG (IL2) cells, a 98.5% decrease in proliferation was seen; at a 90:5:5 ratio, a 93.2% decrease was seen; at a 98 : 1 : 1 mixture, a 52.8% difference was seen compared to the control. No significant trend was observed for the coincubation of mixtures of MNNG (NV) and MNNG (IL2) cells; proliferation levels remained around 50% of the control or better. Mixtures of MNNG (STK) and MNNG (IL2) shows that the IL2-transduced cells do not constitute a significantly different target cell for the bystander factor than do the MNNG (NV) cells (table 4 and fig 9.)

Table 4: Percent Inhibition at Varying Ratios of Pretransduced MNNG cells

Percent Vector-Positive Cells	Expected Inhibition	STK : NV Cells	STK / IL2 : NV Cells	IL2 : NV Cells	STK : IL2 Cells
100	100	99.4	99.3	38.7	98.7
50	50	99.4	98.5	51.5	99.1
25	25	98.6	96.4	41.5	98.2
10	10	94.9	93.2	27.7	96.8
5	5	87.5	87.2	31.8	95.1
2	2	84.2	52.8	24.8	90.2
1	1	40.7	2.3	14.4	71.0
0	0	0	0	0	0

FIGURE 9: MNNG In Vitro Bystander Assay

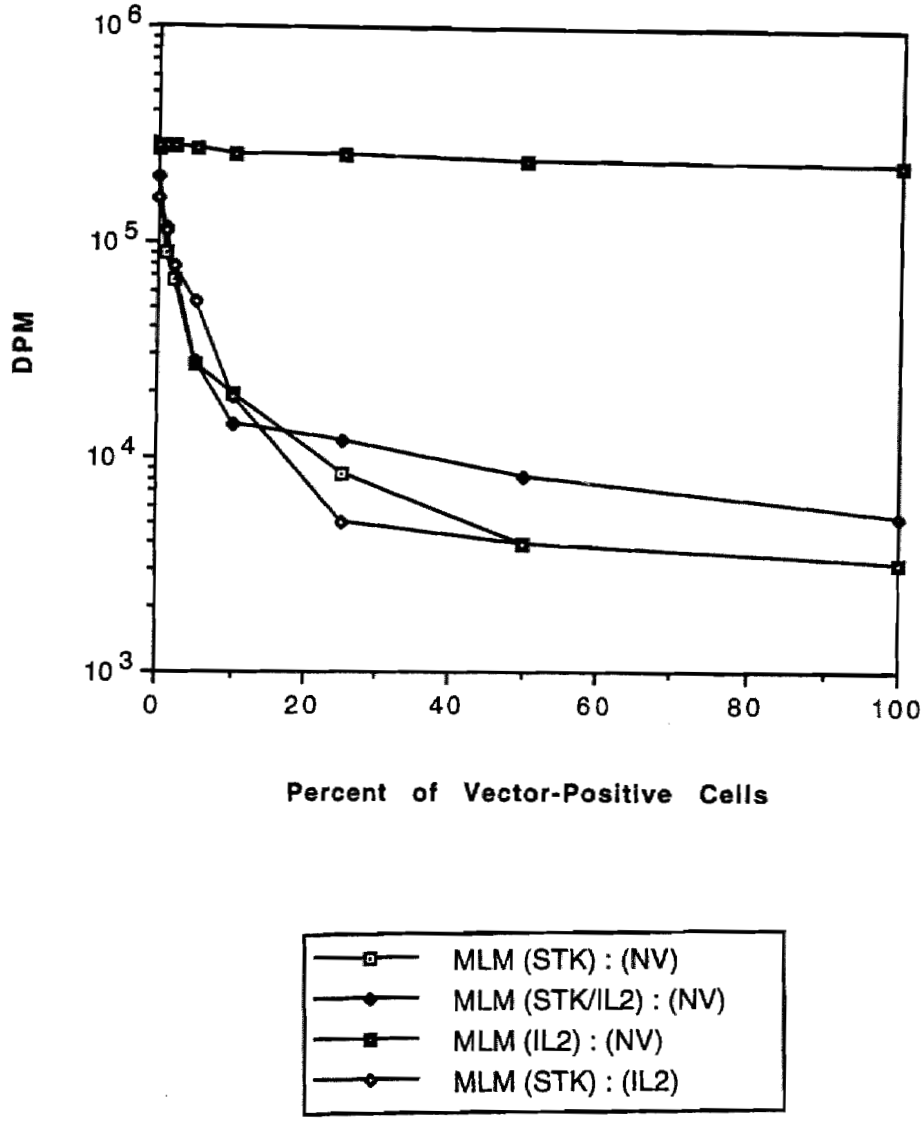


MLM. Coincubation of MLM (STK) and MLM (NV) cells resulted in a substantial decrease in proliferation at each ratio tested. An equal mixture of these two cell types resulted in a 98.51% inhibition compared to the control of MLM (NV) cells alone. With a mixture containing 90% MLM (NV) cells and 10% MLM (STK) cells, an 89.9% decrease in proliferation was observed. With a mixture containing 2% MLM (STK) cells and 98% MLM (NV) cells, a 76.1% decrease in proliferation was seen. When half of the MLM (STK) cells are exchanged for MLM (IL2) cells, no significant increase in inhibition is observed. At a 50:25:25 percent mixture of MLM (NV), MLM (STK) and MLM (IL2) cells, a 95.8% inhibition was observed. At a 90 : 5 : 5 mixture, a 85.8% decrease in proliferation was seen. With 98% MLM (NV) cells and 1% each of the transduced cells, a 42.3% decrease in proliferation was observed. There was no significant difference between the counts obtained from MLM (NV) cell alone and mixtures of MLM (NV) and MLM (IL2) cells. Using MLM (IL2) cells as a target for MLM (STK) cells resulted in counts which were not significantly different from those obtained using MLM (NV) cells as a target. See below (table 5 and fig. 10).

Table 5: Percent Inhibition at Varying Ratios of Pretransduced MLM cells

Percent Vector-Positive Cells	Expected Inhibition	STK : NV Cells	STK / IL2 : NV Cells	IL2 : NV Cells	STK : IL2 Cells
100	100	98.8	97.3	13.3	98.0
50	50	98.5	95.8	8.1	98.5
25	25	96.8	94.1	16.6	96.9
10	10	92.7	92.9	9.7	87.9
5	5	89.9	85.9	4.1	66.8
2	2	85.2	61.1	0.4	52.2
1	1	66.6	42.3	0.8	29.7
0	0	0	0	0	0

FIGURE 10: MLM In Vitro Bystander Assay

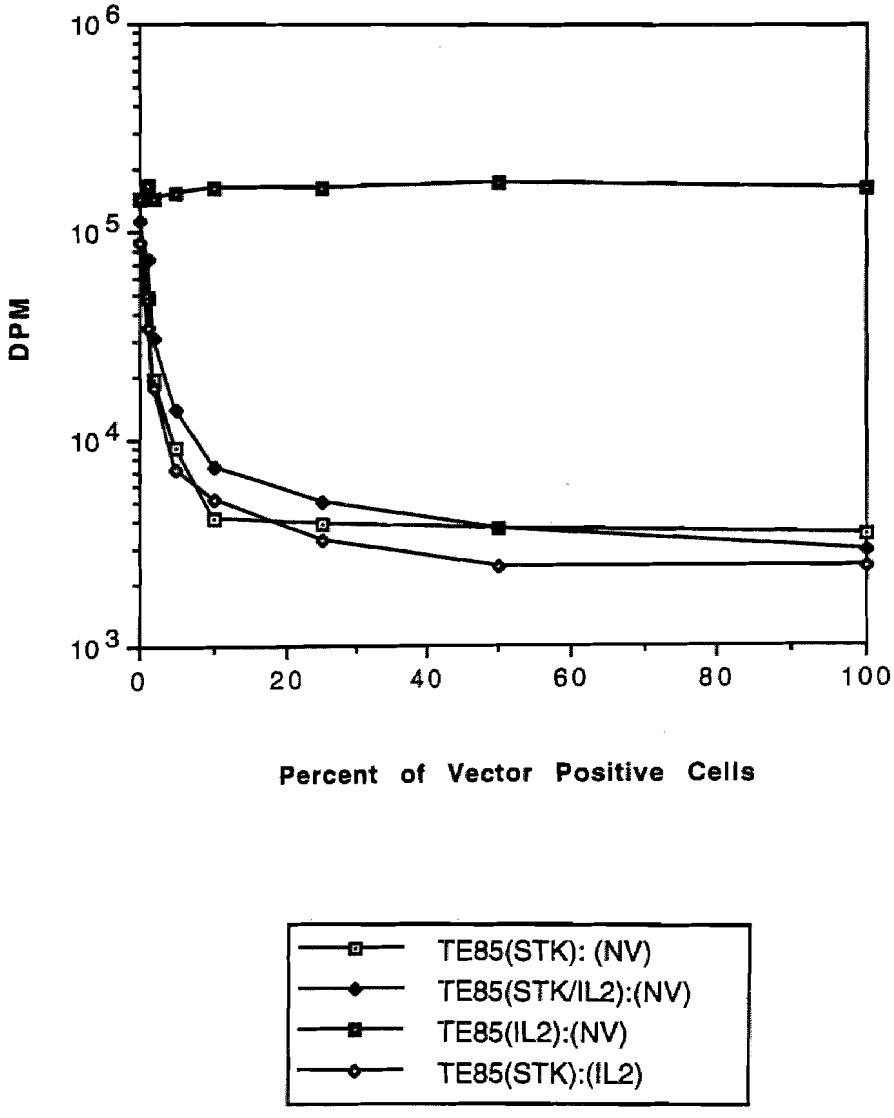


TE85. Coincubation of TE85 (NV) and TE85 (STK) cells resulted in a decreased level of proliferation at each ratio. At an equal mixture of TE85 (NV) and TE85 (STK) cells, the result was a 97.5% inhibition versus TE85 (NV) cell alone. With a mixture containing 90% TE85 (NV) and 10% TE85 (STK) cells, a 97.1% decrease was seen. At a ratio of 98% TE85 (NV) and 2% TE85 (STK), a 66.9% decrease was observed. Exchanging half of the TE85 (STK) cells for TE85 (IL2) cells did not significantly change the effect. At a 50 : 25 : 25 ratio, a 96.8% decrease in proliferation was seen. At a 90 : 10 : 10 ratio, a 93.7% inhibition resulted. At 98% TE85 (NV) and one percent of each transduced cell type, a 72.7% decrease in proliferation was observed. TE85 (IL2) was no different as a target than wild type cells, and there was no significant difference in the counts obtained at the different mixtures of TE85 (IL2) and TE85 (NV) cells. See below (table 6 and fig 11).

Table 6: Percent Inhibition at Varying Ratios of Pretransduced TE85 cells

Percent Vector-Positive Cells	Expected Inhibition	STK : NV Cells	STK / IL2 : NV Cells	IL2 : NV Cells	STK : IL2 Cells
100	100	97.6	97.5	6.7	97.3
50	50	97.4	96.8	0	97.2
25	25	97.2	95.6	6.9	96.4
10	10	97.1	94.7	6.1	94.2
5	5	95.7	87.6	12.4	91.9
2	2	86.7	72.7	16.0	80.0
1	1	66.9	64.8	4.2	60.4
0	0	0	0	18.0	0

FIGURE 11: TE85 In Vitro Bystander Assay

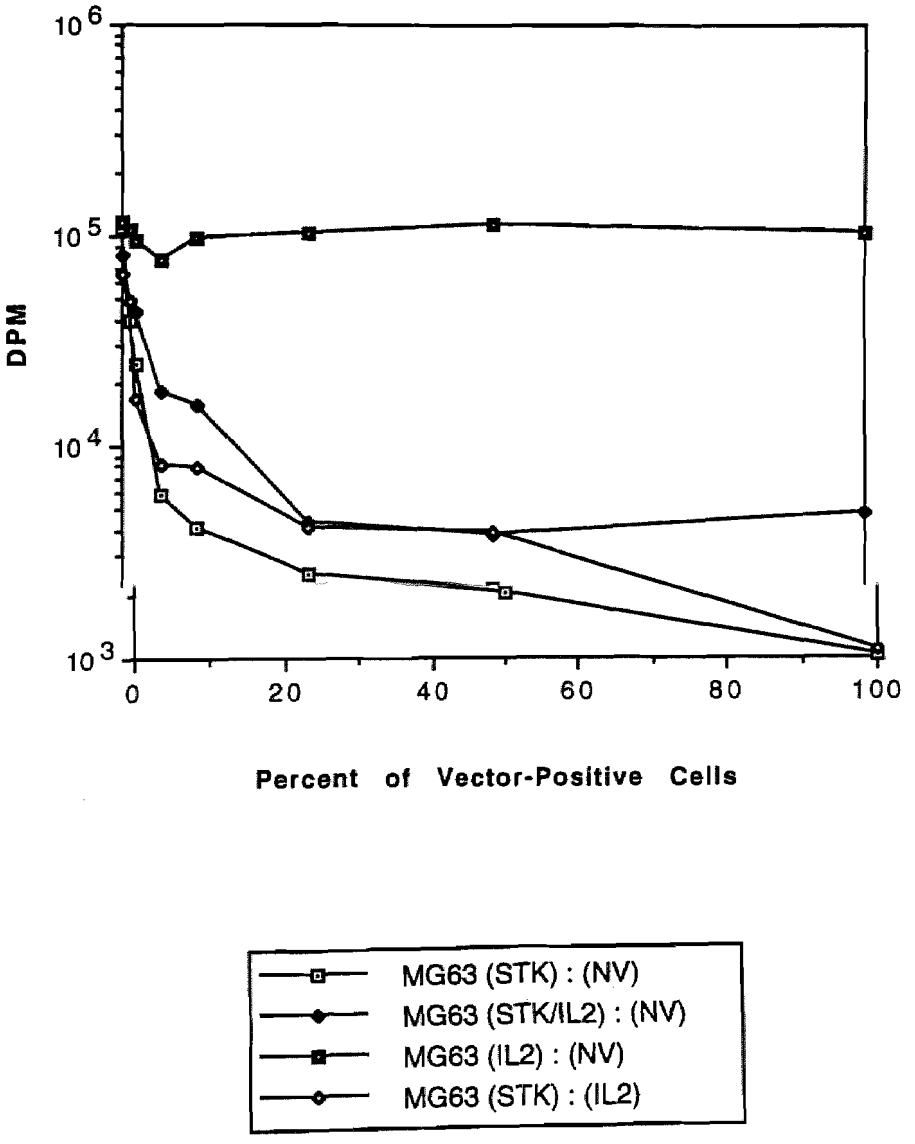


MG63. Coincubation of MG63 (NV) and MG63 (STK) cells resulted in a decreased level of proliferation at each ratio. At an equal mixture of MG63 (NV) and MG63 (STK) cells, the result was a 98.14% inhibition versus MG63 (NV) cell alone. With a mixture containing 90% MG63 (NV) and 10% MG63 (STK) cells, a 94.6% decrease was seen. At a ratio of 98% MG63 (NV) and 2% MG63 (STK), a 77.5% decrease was observed. Exchanging half of the MG63 (STK) cells for MG63 (IL2) cells did not significantly change the effect, although a lower efficiency of inhibition was seen at each ratio. At a 50 : 25 : 25 ratio, a 95.3% decrease in proliferation was seen. At a 90 : 10 : 10 ratio, a 77.2% decrease in proliferation resulted. At 98% MG63 (NV) and one percent of each transduced cell type, a 45.15% inhibition was observed. There was no significant difference in the counts obtained at the different mixtures of MG63 (IL2) and MG63 (NV) cells, and MG63 (IL2) was not significantly different as a target than wild type cells. See below (table 7 and fig. 12).

Table 7: Percent Inhibition at Varying Ratios of Pretransduced MG63 cells

Percent Vector-Positive Cells	Expected Inhibition	STK : NV Cells	STK / IL2 : NV Cells	IL2 : NV Cells	STK : IL2 Cells
100	100	99.1	94.1	9.8	98.3
50	50	98.1	95.3	2.3	94.2
25	25	97.8	94.5	9.3	93.8
10	10	96.3	80.1	15.6	88.0
5	5	94.7	77.2	33.1	88.7
2	2	77.5	45.1	18.9	74.8
1	1	63.5	38.9	6.4	25.6
0	0	0	0	0	0

FIGURE 12: MG63 In Vitro Bystander Assay

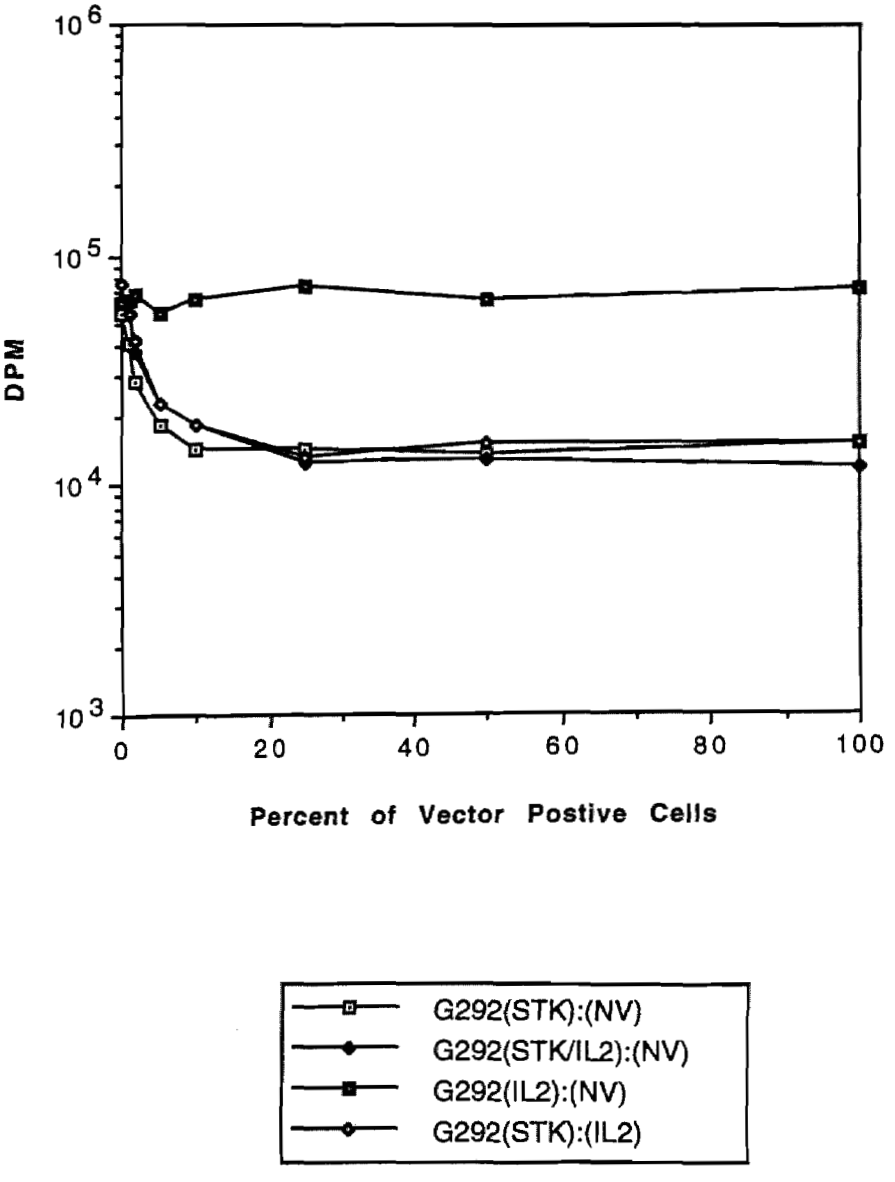


G292. This cell line showed the lowest response to the bystander effect. Moreover, the counts obtained for the first three treatment categories were not significantly different between the ratios of 0-95% target cells. Coincubation of G292 (NV) cells with 50%, 10%, and 2% G292 (STK) cells resulted in a decrease in proliferation of 75.32%, 74.14%, and 49.35% respectively. Coincubation of G292 (NV) cells with 25%, 5%, and 1% each of G292 (STK) and G292 (IL2) cells resulted in a decrease in proliferation of 79.8%, 71.8%, and 40.1% respectively. Coincubation of mixtures of G292 (IL2) cells and G292 (NV) cells resulted in counts which were not significantly different from one another, and G292 (IL2) was not significantly different as a target than wild type cells. See below (table 8 and fig. 13).

Table 8: Percent Inhibition at Varying Ratios of Pretransduced G292 cells

Percent Vector-Positive Cells	Expected Inhibition	STK : NV Cells	STK / IL2 : NV Cells	IL2 : NV Cells	STK : IL2 Cells
100	100	72.7	81.7	2.0	79.9
50	50	75.3	79.8	11.7	80.1
25	25	74.4	80.7	0	82.3
10	10	74.1	71.7	12.5	75.6
5	5	67.0	64.2	23.5	69.9
2	2	48.3	40.1	7.4	43.9
1	1	24.6	3.5	10.1	26.9
0	0	0	0	0	0

FIGURE 13: G292 In Vitro Bystander Assay



MNNG (Trial 2). A more rigorous assay was designed to confirm the trend in the bystander effect seen in the MNNG line above, using twelve replicates per mixture, with mixtures set up as a geometric progression. Two categories of mixtures were made: MNNG (STK) and MNNG (NV) cells, and MNNG (STK), MNNG (IL2), and MNNG (NV) cells. In the first category, MNNG (STK) and MNNG (NV) cells were plated at the following ratios: 100:0, 1:99, 2:98, 4:96, 8:92, 16:84. 32:68, 64:36, and 0:100, at an overall concentration of $5x10^4$ cells per ml. Results are shown below (table 9 and fig. 14).

Table 9: Absence of HS-tk / IL2 Synergy as Shown by Percentage Inhibition at Varying Ratios of Wild-Type, Tk-Transduced, and IL2-Transduced MNNG Cells.

	Percent Vector-Positive Cells								
	0	1	2	4	8	16	32	64	100
STK : NV	0	46.6	86.2	91.8	95.1	97.3	98.2	98.1	98.0
STK / IL2 : NV	0	0	7.7	88.0	91.0	94.9	96.9	97.7	98.4

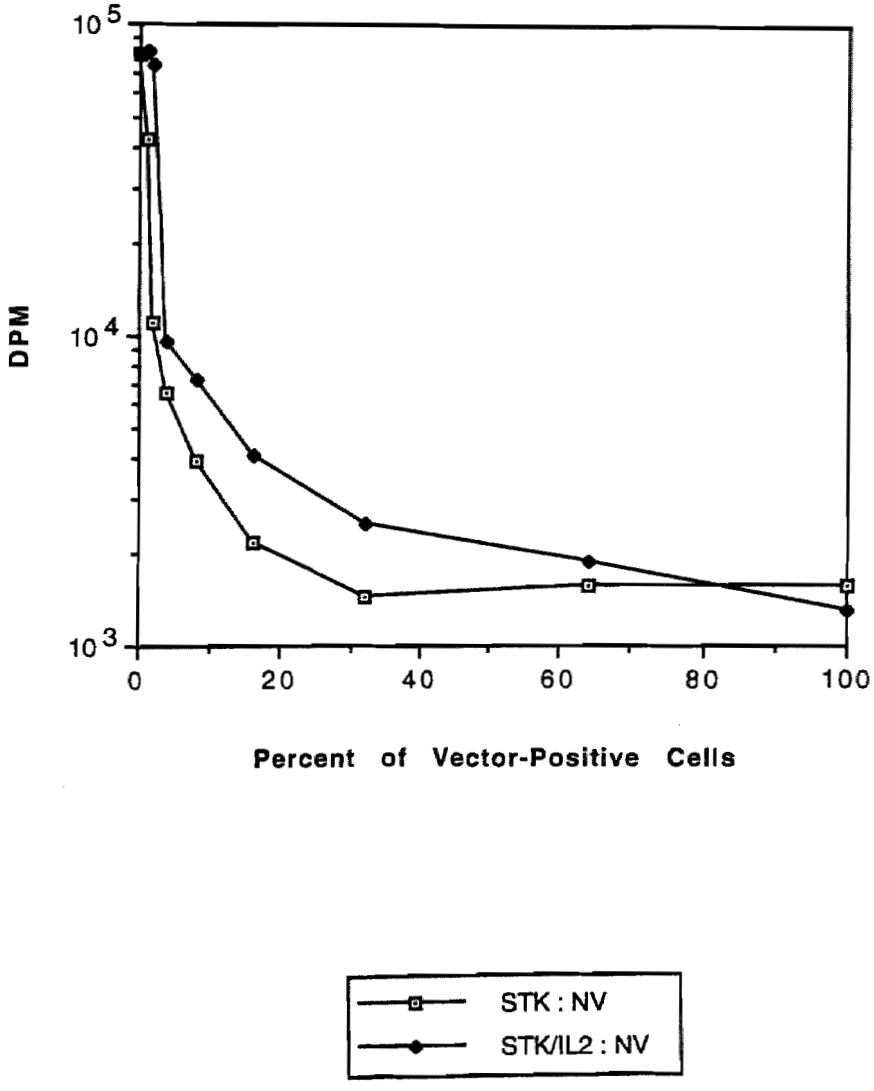
The wells having only MNNG (NV) had the highest count (79,940 +/- 11,583 DPM), indicating that the greatest number of cells survived to replicate their DNA in this group. Thereafter, an increasing percentage of MNNG (STK) cells resulted in decreased DPM, as more cells were subjected to GCV-mediated inhibition. With 1% MNNG (STK), a 46.6% decrease (42,683 +/- 21,766 DPM) in proliferation was seen when compared to the MNNG (NV) alone. With 2% MNNG (STK), an 86.2% decrease (11,058 +/- 1975 DPM) was seen. With 4% MNNG (STK), a 91.8% decrease in proliferation (6585 +/- 1086 DPM) was observed. The mixture of 8% MNNG (STK) cells with 92% wild type resulted in a 95.1% inhibition (3918 +/- 630 DPM). With 16% MNNG (STK) cells, a 97.3% decrease (2163 +/- 288 DPM) was seen. With 32% MNNG (STK) cells, a 98.2% decrease (1450 +/- 159 DPM) was observed. Between each of these successive treatment groups, the DPM are significantly different ($p < 0.05$). However, there was no significant difference seen between the 32% and 64% MNNG (STK) groups.

To evaluate the synergistic potential of MNNG (IL2), the same ratios of cells as above were plated, but using an equal mixture of MNNG (STK) and MNNG (IL2) cells

where previously MNNG (STK) cells had been used singularly. A mixture of 98% wild type cells and 1% each MNNG (STK) and MNNG (IL2) resulted in a slight (7.7%) decrease in proliferation (73,791 +/- 19,371 DPM) from the 100% wild type control. A mixture of 96% wild type cells and 2% each MNNG (STK) and MNNG (IL2) resulted in a substantial (88%) decrease in proliferation (9574 +/- 1406 DPM). At a 92 : 4 : 4 mixture, a 91.0% decrease in proliferation (7187 +/- 517 DPM) was observed. Doubling the number of transduced cells to 8% each resulted in a 94.9% decrease in proliferation (4080 +/- 501 DPM). A mixture of 16% of each transduced cell type and 68% wild type cells resulted in a 96.9% decrease in proliferation (2484 +/- 461 DPM) over the control. A mixture of 32% each of MNNG (STK) and MNNG (IL2) along with 36% wild type cells yielded a 97.7% decrease in proliferation (1852 +/- 159 DPM). The values obtained from the 2:2:96 mixtures through the 32:32:36 mixtures are all significantly different ($p < 0.05$) from one another.

The matter of the HS-tk and IL2 gene products acting synergistically to inhibit cellular proliferation, addressed by comparing the results between rows 1 and 2 in table 9 above, may be considered from two standpoints: as a constant percentage of tumor cells (i.e., comparing a given number in row 1 with the number directly below in row 2), or as a constant percentage of HS-tk-transduced cells (i.e., comparing a given number in row 1 with the number below and immediately to the right in row 2, because, for example, "8% transduced cells" in row one and "16% transduced cells "in row 2 each contain 8% STK-transduced cells.). In the first case (when the percentage of tumor cells is held constant), the figures are significantly different ($p < 0.05$) between row 1 and row 2; at a given percentage of wild type MNNG cells, an equal mixture of STK-transduced and IL2-transduced cells results in a relatively lower level of inhibition compared with STK-transduced cells and no IL2-transduced cells. In the second case (when the percentage of STK-transduced cells is kept constant between rows 1 and 2), the results obtained from comparing between the two rows are not significantly different, indicating that at a given percentage of STK-transduced cells, the level of growth inhibition is unaffected by the presence of IL2-transduced cells. Thus, a model of synergy between the HS-tk and IL2 gene products is not supported.

Figure 14: MNNG In Vitro Bystander Assay (Trial 2)



In Vivo

Preliminary Studies. To test which of the original four cell lines (MNNG, TE85, MG63, and G292) would be tumorigenic in nude mice, two concentrations cells (each in 0.1 ml HBSS) were injected both subcutaneously ($1x10^7$ and $2 x10^7$ cells) and into the vascular system through the ventrolateral tail vein ($2x10^6$ and $5x10^6$ cells). Three mice were injected at each level in each experimental group, for a total of 24 mice. During tail vein injections, a total of four mice died (one in a low dose group and three in a high dose group). Several others experienced whole-body twitching but recovered fully within a matter of minutes. All subcutaneous injections were administered without incident.

Of the subcutaneous groups, only MNNG was tumorigenic. No mice receiving tail-vein injection of any cell line developed disseminated tumors, though mice receiving MNNG intravenously did develop local tumors at the injection site.

The novel fifth osteosarcoma cell line (MLM) was a metastatic lung tumor from a nude mouse receiving an intratibial implant of MNNG cells. This line produced subcutaneous growth similar to that of the parental MNNG line, and yielded no metastasis when injected intravenously.

Threshold for MNNG tumorigenicity. Varying concentrations of MNNG cells (0.05 ml in HBSS) were injected subcutaneously to nude mice. No tumors developed in ten mice injected with $5x10^4$ cells. In mice injected with $1x10^5$ cells, 17.6% (3/17) developed tumors. All mice receiving $2x10^5$ cells or more (N = 380) developed tumors. See below (table 10).

Table 10 : Cellular Threshold for Tumorigenicity of MNNG Human Osteosarcoma in Nude Mice

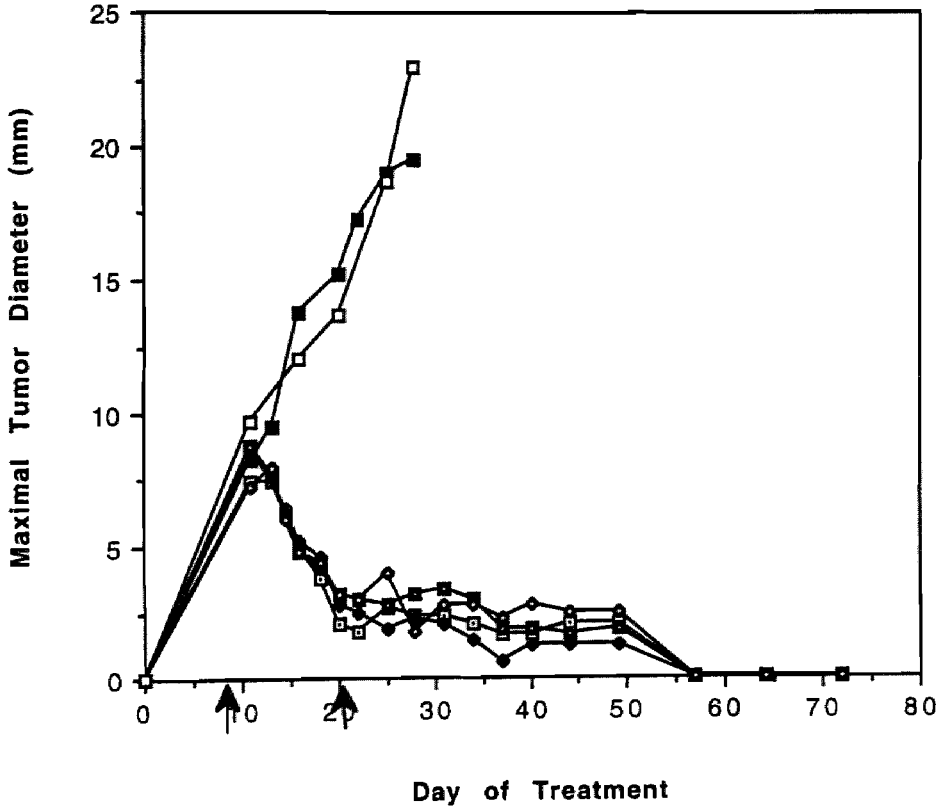
CELL NUMBER	NUMBER OF MICE DEVELOPING TUMORS
5×10^4	0 / 10
1×10^5	3 / 17 (17.6%)
2×10^5	20 / 20
2.5×10^5	21 / 21
5×10^5	88 / 88
8×10^5	32 / 32
2×10^6	144 / 144
5×10^6	69 / 69
1×10^7	3 / 3
2×10^7	3 / 3

Mixture of STK vector-producer cells and MNNG cells. In an effort to determine the relative number of STK vpc needed to completely destroy tumors, twenty mice were divided evenly into four groups. Mice in Group 1 received a subcutaneous injection of a mixture of wild type MNNG cells ($5x10^5$) and STK vpc ($2.5x10^6$), a 1:5 ratio. Mice in Group 2 received a mixture of MNNG ($1x10^6$) and STK vpc ($2x10^6$), a 1:2 ratio. Mice in Group 3 received a 1:1 ratio of each cell type ($1x10^6$ cells each). In Group 4, mice received a mixture of MNNG cells ($1x10^6$) and STK vpc ($5x10^5$), a 2:1 ratio. Beginning on day eight and continuing through day 19, all mice received injections of GCV (3 mg in 1 ml HBSS) twice daily. Measurements of maximal tumor diameter were taken daily throughout GCV treatment.

Control groups consisted of seven mice which received wild type MNNG cells ($5x10^5$) alone; four were treated with GCV, and the other three were given saline injections instead. Also, 12 mice were divided into four groups; these mice received identical mixtures of MNNG (NV) and STK vector producer cells as above, but were given saline injections instead of GCV.

Figure 15 shows the results of this experiment (in this and all subsequent graphs, arrows depict the range of GCV treatment). All mice receiving mixtures of MNNG (NV) and STK vpc developed tumors, which began to regress within two to four days after the commencement of GCV injections. All mice in the experimental groups were completely cured, except for one mouse in the group receiving the fewest number of STK vpc, which developed regrowth. All mice in control groups experienced progressive tumor growth until sacrifice was necessary at day 28. Tumor growth in control mice receiving mixtures of MNNG cells and STK vpc and no GCV (not shown) was not significantly different from that in mice receiving only MNNG cells and no GCV. There was also no significant difference in tumor growth between control mice receiving GCV and control mice not receiving GCV.

FIGURE 15: Mixtures of STK Vpc and MNNG (NV) Human Osteosarcoma Cells



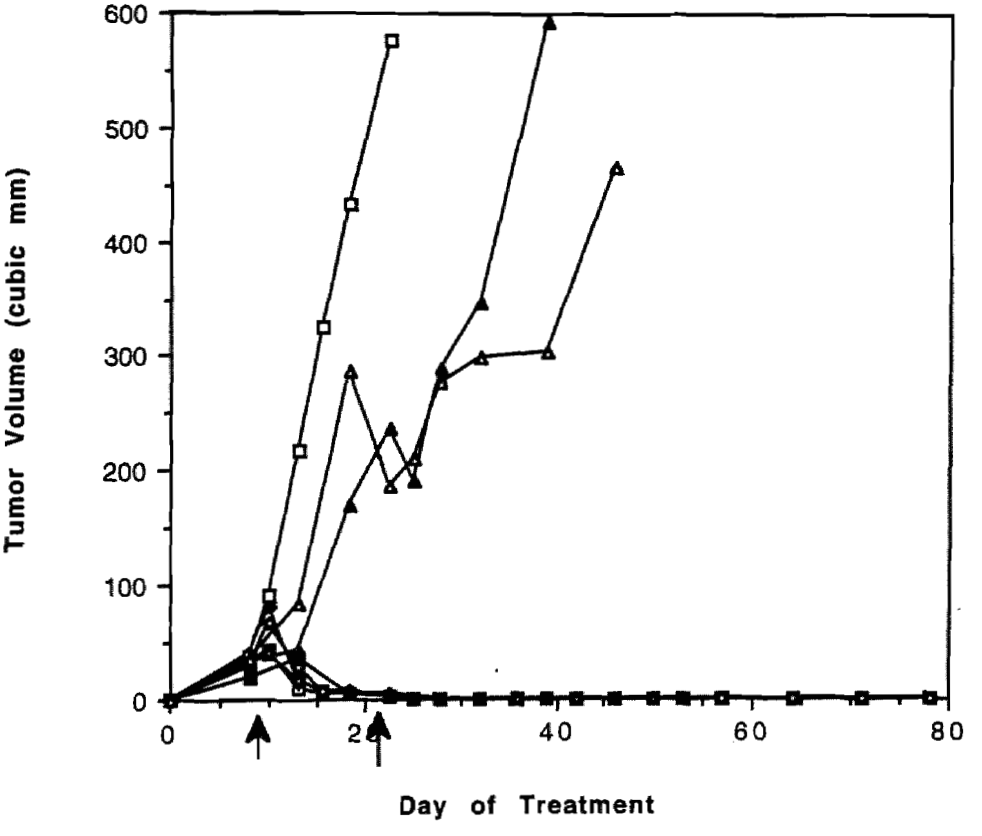
***In Vivo* Bystander Assay.** (Fig. 16-18). A total of 26 groups were formulated, each testing different ratios of MNNG (NV) cells to transduced cells [MNNG (STK) and/or MNNG (IL2)] For clarity's sake, group numbers are followed parenthetically by the percentage of transduced cells in the inoculum From three to 11 mice were assigned to each group, for a total of 144 mice. Each mouse received a total of $2x10^6$ cells as a subcutaneous injection. Except as noted, all mice developed tumors which increased in size from day one to day ten. From day eight to day 19, mice in groups 1-17 received GCV (3 mg in 1 ml HBSS) twice daily. Mice in groups 18-26 did not receive GCV. All mice were monitored for up to ten weeks following completion of GCV therapy. Three mice in separate groups (groups 3 (IL2 100), 10 (STK12.5), and 12 (IL2 12.5)) showed regrowth after having been apparently cured; data from these animals is not included in the graphs showing average tumor volume. Several other groups (generally IL2 groups without GCV) contained multiple mice showing regrowth; measurements from these mice were included graphically. Five mice died from GCV toxicity (defined below) during the course of injections; these mice are included in the averaged data. All healthy mice were followed for a minimum of six weeks prior to termination of the experiment.

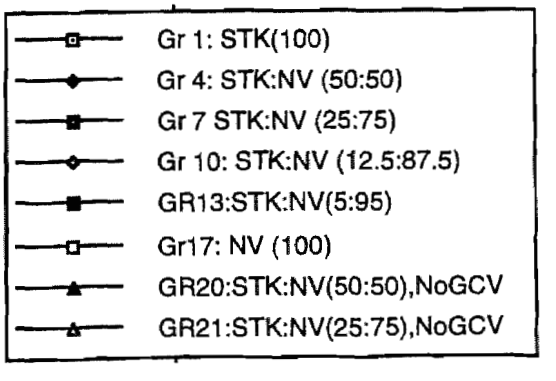
STK and NV mixtures. Figure 16 below shows graphically the results from all groups receiving MNNG (STK) cells, including controls. Table 11 below shows the percentage of mice cured in each group.

Table 11: *In vivo* Bystander Experiment: STK Groups

GROUP	Number of Mice Cured	Percent
GR 1 (STK 100)	5 / 5	100
GR 4 (STK 50)	10 / 10	100
GR 7 (STK 25)	11 / 11	100
GR 10 (STK 12.5)	10 / 11	91
GR 13 (STK 5)	3 / 3	100
GR 17 (STK 0)	0 / 5	0

FIGURE 16: In Vivo Bystander Experiment: All STK Groups





Group 1 (STK100) contained five mice, each of which developed tumors and were cured by day 28. Group 4 (STK50) contained ten mice, all of which developed tumors that were cured by day 28. Group 7 (STK25) contained 11 mice, all of which developed tumors that were cured by day 28. Group 10 (STK12.5) had 11 mice; ten of these mice (91%) were cured by day 28. The other mouse had no palpable tumor from days 22 to 36, then experienced regrowth (8 mm^3 by day 43 up to a terminal volume of 360 mm^3). Group 13 (STK 5) contained three mice, all of which developed tumors that were cured by day 25.

Group 17 (STK 0) was a control group containing five mice which received only MNNG (NV) cells; all of these mice received the standard GCV cycle, and showed progressive growth (to a final average of 580 mm^3) until euthanasia was necessary at day 28. Two other control groups not receiving GCV were present in the STK-containing collection. Group 20 (STK50) contained three mice, each of which showed progressive growth (to a final average of 595 mm^3) and was sacrificed on day 39. Group 21 (STK25) also contained three mice, each of which showed progressive growth (to a final average of 460 mm^3) until euthanasia at day 46.

IL2 and NV mixtures. Figure 17 depicts the results obtained from the ten groups receiving mixtures of MNNG (IL2) cells and MNNG (NV) cells; table 12 below shows the percentage of cured mice by group.

Table 12: *In vivo* Bystander Experiment: IL2 Groups

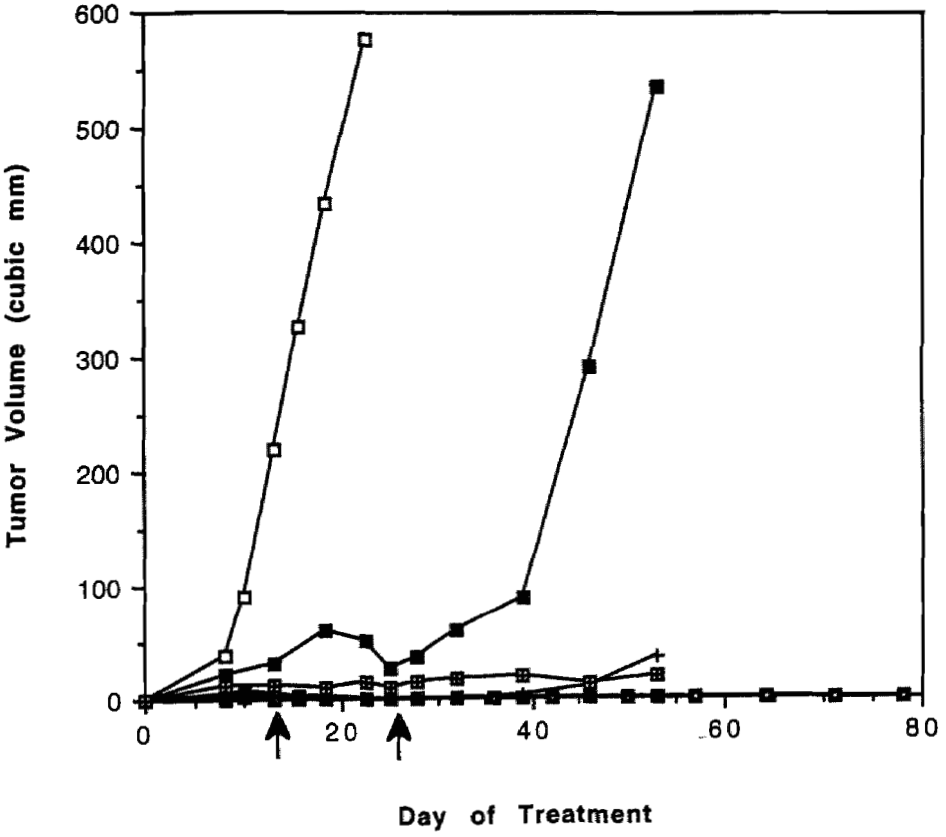
GROUP	Number of Mice Cured	Percent
GR 3 (IL2 100)	4 / 5	80
GR 6 (IL2 50)	5 / 5	100
GR 9 (IL2 25)	5 / 5	100
GR 12 (IL2 12.5)	4 / 5	80
GR 15 (IL2 5)	1 / 5	20
GR 17 (IL2 0)	0 / 5	0
GR 18 (IL2 100: $2x10^6$), No GCV	3 / 3	100
GR 19 (IL2 100: $5x10^6$), No GCV	3 / 3	100
GR 22 (IL2 50), No GCV	2 / 3	67
GR 23 (IL2 25), No GCV	2 / 3	67
GR 24 (IL2 10), No GCV	1 / 4	25

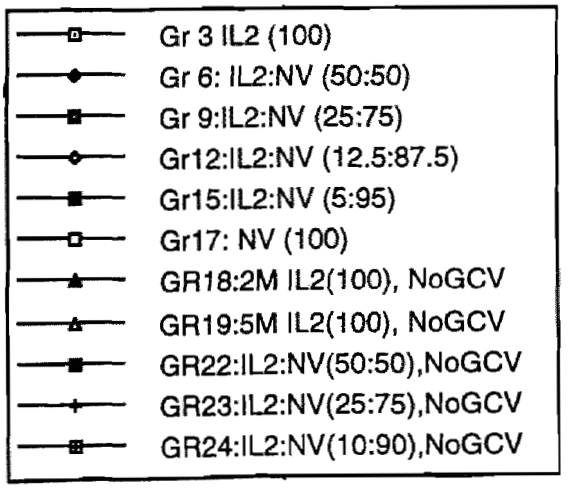
Groups 3, 6, 9, 12, 15, and 17 each contained five mice, all of which received GCV; mice in groups 18, 19, 22, 23, and 24 did not receive GCV. Mice in Group 3 (IL2 100) received subcutaneous injections of only MNNG (IL2) cells and GCV. Each mouse showed small tumor growth (to an average maximal volume of 3.56 mm^3 by day ten). Four of these mice (80%) were cured completely by day 24. The fifth mouse appeared to be tumor-free, then showed small but steady regrowth (3 mm^3) from days 64-78. Mice in Group 6 (IL2 50) all showed small tumor growth (average maximal volume of 3.14 mm^3 at day ten), followed by total ablation by day 20. Mice in Group 9 (IL2 25) each developed a small tumor (maximum average volume of 6.07 mm^3 by day 10) which was eliminated completely by day 20. Mice in Group 12 (IL2 12.5) each initially developed a small tumor (average maximal volume of 4.92 at day 13); tumors on four of these mice

(80%) disappeared permanently by day 28. The other mouse showed an 80% regression in tumor volume, but the tumor then expanded progressively from day 37-64, to a final volume of 377 mm^3. Mice in Group 15 (IL2 5) each developed tumors; by day 18, the average tumor volume was 60.32 mm^3. Thereafter, one mouse (20%) was cured completely by day 46 and showed no regrowth. The other four mice showed a 54% average regression ($p < 0.05$) in tumor mass, followed by progressive growth to an average maximal volume of 536 mm^3 by day 53. Two of these mice showed no visible tumor from days 28 - 35, but then developed regrowth.

The remaining IL2 groups did not receive GCV. Group 18 (IL2 100) contained three mice, each which received an implant of $2x10^6$ MNNG (IL2) cells. Only one of these mice showed any tumor growth whatsoever, and this disappeared by day ten. Group 19 (IL2 100) contained three mice, each of which received $5x10^6$ MNNG (IL2) cells. Each of these mice developed a small tumor (average maximal volume of 3.66 mm^3 by day 8), which was eliminated by day 18. The following three groups received mixtures of MNNG (IL2) and MNNG (NV) cells and no GCV. Group 22 (IL2 50) contained three mice, each of which developed small tumors (maximum average volume of 4.19 mm^3 at day 8) that dissipated by day 25. Two mice (67%) proved to be cured permanently. The third developed small but persistent regrowth (2 mm^3 by day 53). Group 23 (IL2 25) contained 3 mice, each of which showed small tumor growth (average maximal volume of 4.36 mm^3 by day 8) then complete regression. Two mice (67%) were cured permanently. The other mouse developed substantial regrowth (100 mm^3 by day 53). Group 24 (IL2 10) contained 4 mice. One mouse (25%) was cured completely by day 28. The other three showed some regression then progressive growth (average terminal volume of 27 mm^3 on day 57).

FIGURE 17: In Vivo Bystander Experiment: All IL-2 Groups



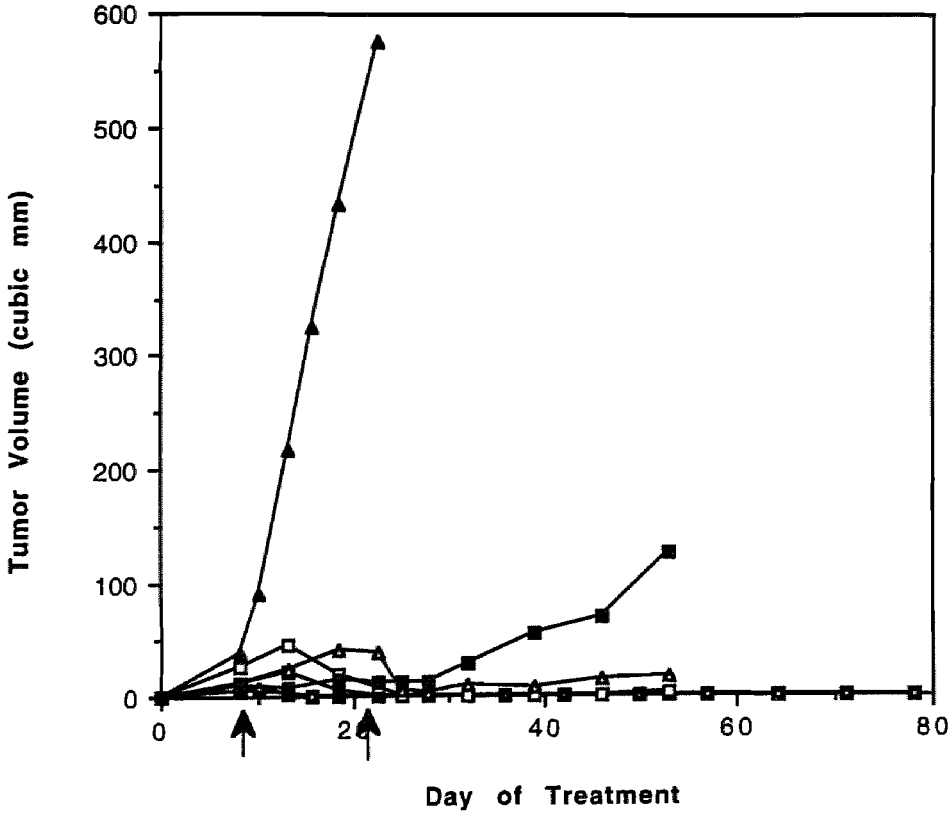


STK, IL2 and NV mixtures. In these groups, MNNG (STK) and MNNG (IL2) cells were introduced in equal proportions along with varying levels of MNNG (NV) cells. The total number of transduced cells is designed in the notations that follow. Figure 18 shows the results obtained from the eight groups that received mixtures of all three cell types. Table 13 below shows the number of cured mice by group.

Table 13: *In vivo* Bystander Assay: STK / IL2 Groups

GROUP	Number of Mice Cured	Percent
GR 2 (STK/IL2 100)	5 / 5	100
GR 5 (STK / IL2 50)	10 / 10	100
GR 8 (STK / IL2 25)	11 / 11	100
GR 11 (STK / IL2 12.5)	11 / 11	100
GR 14 (STK/IL2 5)	4 / 4	100
GR 16 (STK/IL2 1)	2 / 5	40
GR 17 (STK/IL2 0)	0 / 5	0
GR 25 (STK/IL2 25), No GCV	2 / 3	67
GR 26 (STK/IL2 12.5), No GCV	1 / 3	33

FIGURE 18: In Vivo Bystander Experiment
All STK-IL2 Groups



- Gr 2: STK:IL2 (100)
- Gr 5 STK-IL2:NV(50:50)
- Gr 8 STK-IL2:NV (25:75)
- Gr 11: STK-IL2:NV (12.5:87.5)
- GR14:STK-IL2:NV(5:95)
- Gr16:STK-IL2:NV(1:99)
- Gr17: NV (100)
- GR25:STK-IL2:NV(25:75),NoGCV
- GR26:STK-IL2:NV(12.5:87.5)NoGCV

Group 2 (STK/IL2 100) contained five mice, all of which developed small tumors (average maximal volume of 5.97 mm^3 on day eight), that were eliminated by day 20. Group 5 (STK/IL2 50) had ten mice, all of which showed moderate growth (peaking at an average volume of 9.48 mm^3 on day 8), followed by complete regression. One mouse died of GCV toxicity on day 22. Group 8 (STK/IL2 25) contained 11 mice, each of which showed moderate tumor growth (a maximal average volume of 5.61 mm^3 on day 10), then complete regression by day 20. Group 11 (STK/IL2 12.5) had 11 mice, each of showed small tumor growth, peaking on day ten with a average volume of 6.38 mm^3. By day 24, all mice had been completely cured. Group 14 (STK/IL2 5) contained four mice, each of which showed moderate growth, to a maximal average of 20.5 mm^3 by day 13. Each tumor had disappeared by day 20. One mouse in this group died of GCV toxicity. Group 16 (STK/IL2 1) had 5 mice, all of which developed tumors of moderate size, which peaked on day 13 at an average volume of 44.7 mm^3. Two mice died on day 25 due to GCV toxicity. On the other three mice, no visible tumor remained by day 28; one mouse showed small regrowth beginning on day 45. The other two (40%) were cured permanently.

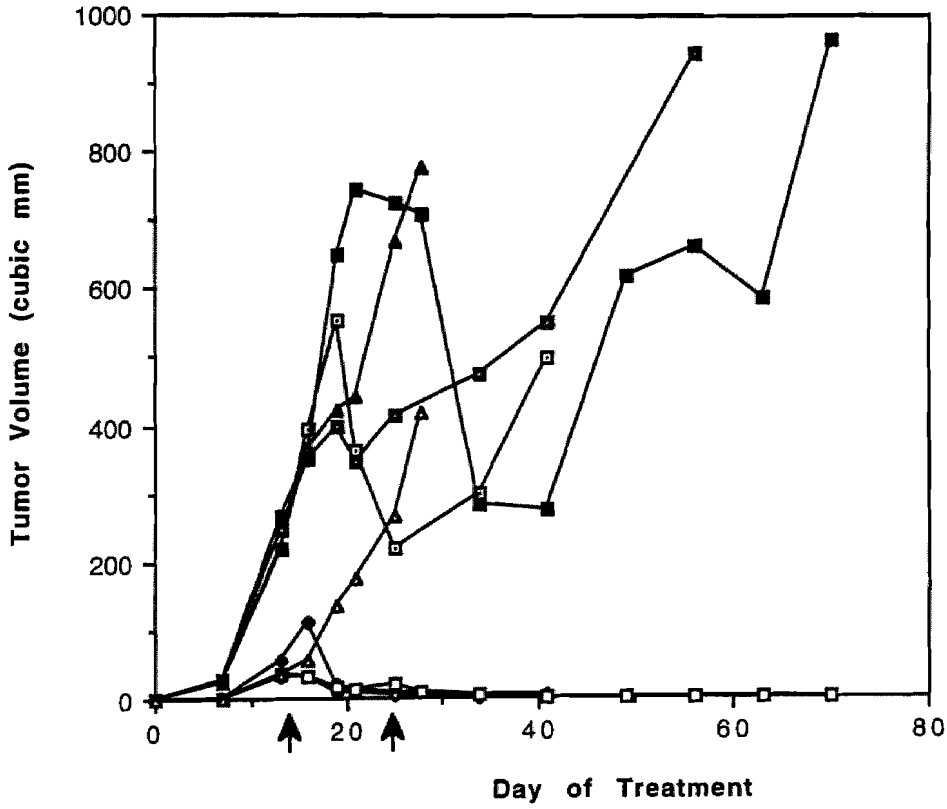
Two control groups received a combination of all three cell types but no GCV. Each of the three mice in Group 25 (STK/IL2 50); by day 18, the tumors had grown to an average volume of 40.67 mm^3, but then began to shrink. By day 32, two mice (67%) had been cured and showed no regrowth. The other mouse showed a 94.6% regression in tumor size by day 28, but then steady regrowth (to 26.2 mm^3 by day 53). Group 26 (STK/IL2 25) contained three mice. One of these mice (33%) was cured by day 25. The other two showed slight (25%) regression, then progressive regrowth (to an average of 189.9 mm^3 by day 53).

Double Tumor Experiment. (Fig 19-23). Forty-two nude mice were divided into four groups. On day one, each mouse received a subcutaneous injection of MNNG (NV) cells ($1x10^6$) on the right ventral side. By day six, all mice had developed dense, nodular tumors (average volume 26.19 +/- 3.12 mm^3). At this time, secondary tumors ($2x10^7$ cells per implant) were induced with MNNG cells which had been transduced with the STK, IL2 or BAG gene. These tumor cells were injected subcutaneously on the left ventral side at a distance of approximately one centimeter from the nearest border of the first tumor. Control mice (the same as those in the bystander experiment) received a similar implant of MNNG (NV) cells, no secondary tumor, and 12 days of GCV. Table 14 below shows the number of animals showing a significant response (i.e., at least a 50% reduction in the volume of the primary MNNG (NV) tumor) in each group. Figure 19 shows the composite results graphically.

Table 14: Double Tumor Experiment

GROUP:	Number of Responders	Percent Responders	Percent Cured
GR 1 (STK)	10 / 12	83	0
GR 2 (STK / IL2)	13 / 18	72	11
GR 3 (IL2)	3 / 6	50	0
GR 4 (BAG)	6 / 6	0	0

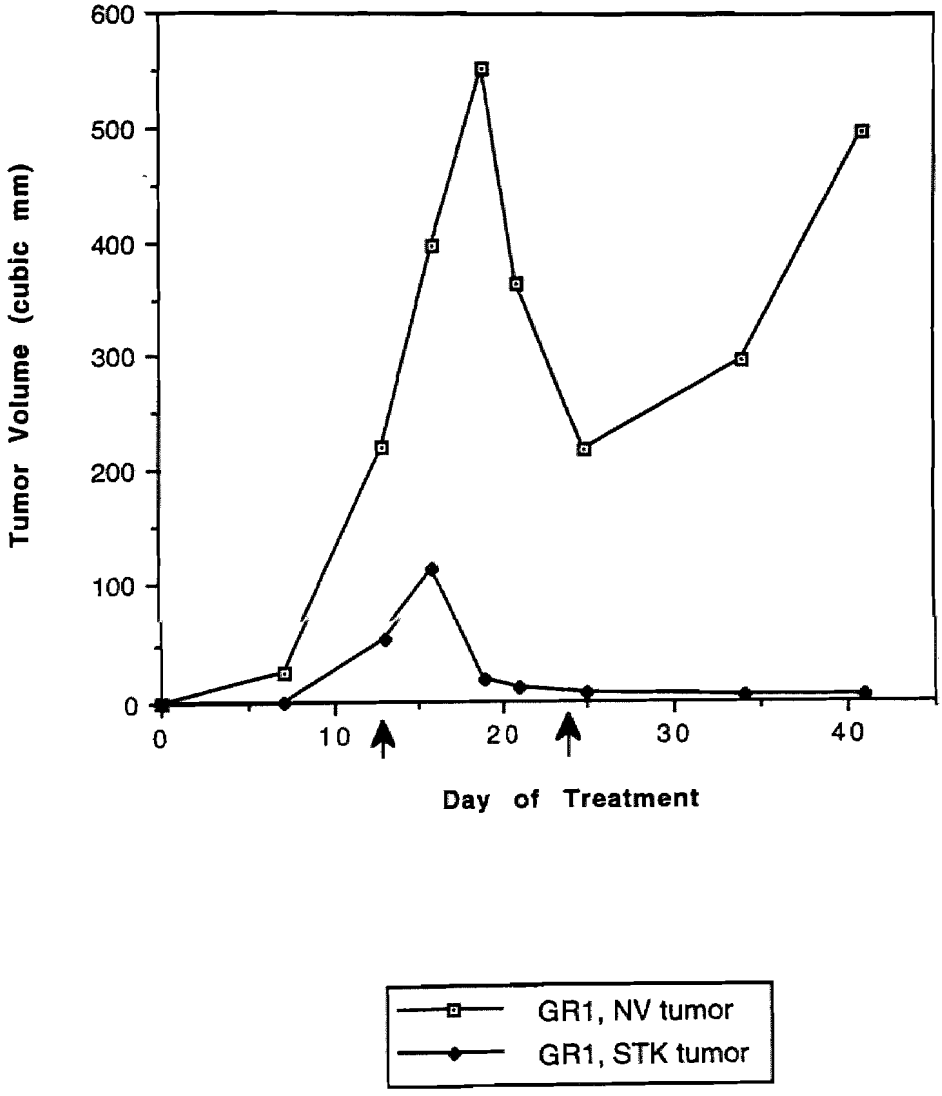
FIGURE 19: Double Tumor Experiment



Group One contained twelve mice, each of which received MNNG (STK) cells ($2x10^7$ each) on day 6. By day 13, the first tumors had expanded considerably, to an average volume of 219.8 +/- 62.3 mm^3. GCV injections were administered twice daily from day 14-25. Ten mice (83%) showed a partial response, experiencing at least a 60% reduction in the primary tumor volume. In these mice, the average reduction was 80.7% (688.5 +/- 260.9 mm^3 to 136.6 +/- 84.2 mm^3), occurring on average from day 18.2 to 25.3. Thereafter, each mouse showed considerable regrowth, to an average volume of 484.4 +/- 353.1 mm^3 (a 356% increase from the average low volume) by day 41 when the experiment was terminated for this group. Two mice were unresponsive and showed progressive growth of the primary tumor. Two toxicity deaths were seen in this group near the end of GCV treatment.

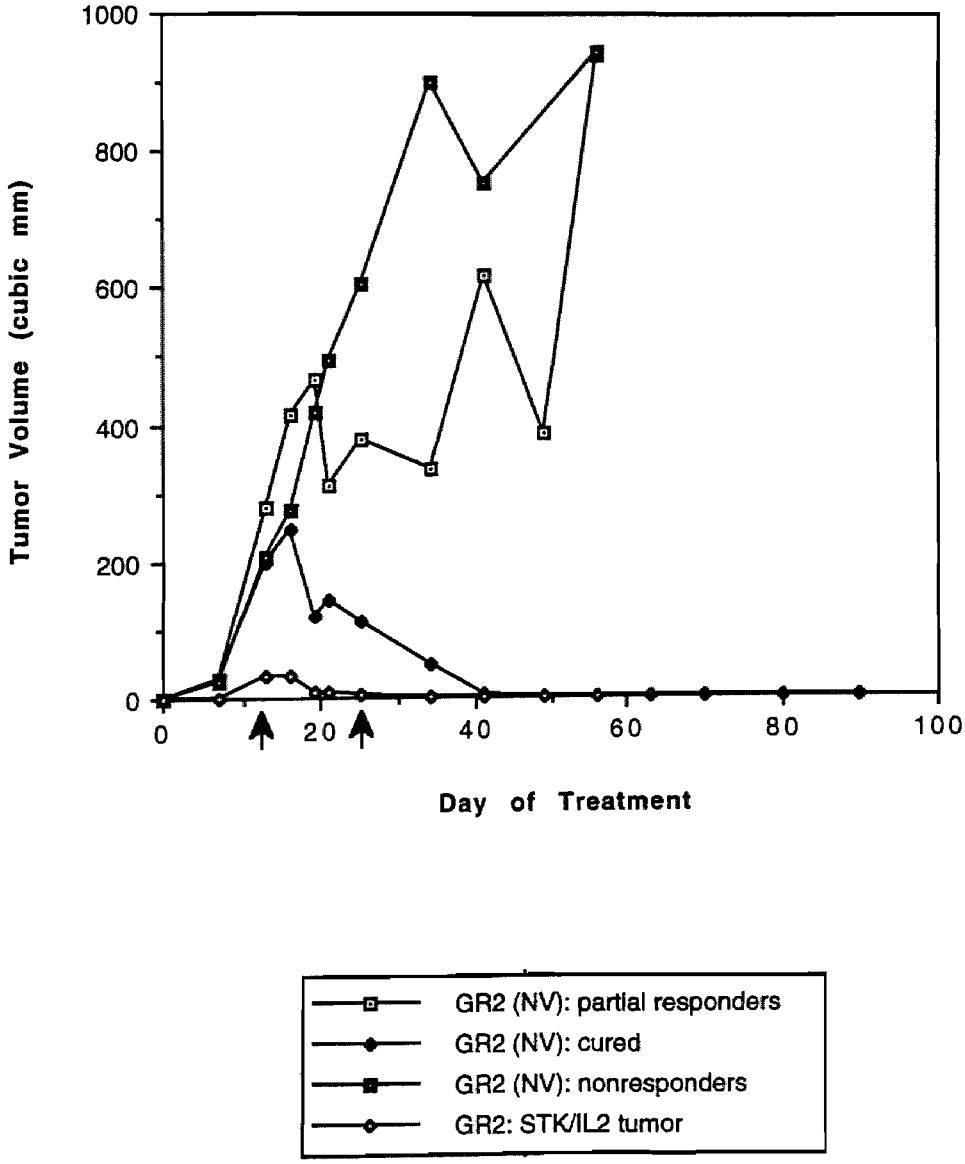
The secondary MNNG(STK) tumors in Group 1 grew to an average maximal volume of 112.2 +/- 59.5 mm^3 by day 16 (i.e., three days into GCV treatment). Thereafter, these tumors shrank quickly. Of the ten mice living by the termination date, seven showed no remaining secondary tumors; the other three had an average tumor volume of 12.83 +/- 11.83 mm^3. See below (fig. 20).

FIGURE 20: Double Tumor Experiment - Group 1
MNNG (STK) Secondary Tumor



Group 2 contained 18 mice, each received a secondary implant of MNNG(STK) and MNNG(IL2) cells ($1x10^7$ each) on day six. By day 13, the initial tumors had increased greatly in volume to an average of 247.1 +/- 59.9 mm^3. GCV was administered from day 14-25. Thirteen mice (72%) showed at least a partial response to therapy, experiencing a 50% or greater reduction in volume of the primary tumor; two of these mice (11%) were cured completely. The 11 partial responders showed an average regression of 82.2% (from 820.77 +/- 307.13 mm^3 to 146.44 +/- 56.02 mm^3), occurring on average from day 20.8 to day 26.2. Each of these mice showed regrowth (to a maximal average of 806.19 +/- 275.7 mm^3) by the completion of the experiment. Tumors on the two cured mice went from maximal volumes of 395.8 mm^3 (day 16) and 150.8 mm^3 (day 19) to zero mm^3 by day 41 and 49. Each mouse was monitored for at least 60 days after the tumor was gone. Five mice (28%) in Group 2 did not show at least a 50% regression in tumor volume; three of these showed no regression whatsoever. Overall, two mice in this group died near the end of GCV treatment. The secondary tumors in Group Two grew to a maximal average volume of 32.41 +/- 11.8 mm^3 by day 16. Each of these tumors had been eradicated by day 41; 12 of 16 (75%) had disappeared by day 34. See below (fig. 21).

FIGURE 21: Double Tumor Experiment - Group 2
MNNG (STK)/MNNG (IL2) Secondary Tumor



Group Three contained six mice, each of which received a secondary implant of MNNG (IL2) cells. By day 13, the first tumors had grown to an average volume of 266.7 +/- 102.5 mm^3. GCV treatment was administered from day 14-25. Three mice (50%) showed a partial response to therapy. One mouse showed a 89.7% decrease in tumor volume (1590 mm^3 on day 28 to 163.4 mm^3 on day 41). The two other mice showed even greater response levels: 98.5% reduction (1246 mm^3 to 18.9 mm^3) and 99.8% reduction (678.6 mm^3 to 1.05 mm^3), each between days 21 and 49. However, all three of these mice developed massive regrowth (to an average of 867 mm^3) by the end of the experiment. The other three mice did not show a significant decrease in the volume of the first tumor; one mouse died near the end of GCV treatment and had failed to show any evidence of regression. The secondary tumors in Group Three grew to an average maximal volume of 40.6 +/- 22.6 mm^3 between days 16 and 19, and had all vanished by day 49 in surviving mice. See below (fig. 22)

Group 4 contained six mice, each of which received a secondary implant of MNNG (BAG) cells. By day 13, the first tumors had grown to an average volume of 224.7 +/- 137.6 mm^3. All of these mice received the standard regimen of GCV therapy. Each primary tumor grew progressively to a maximal volume of 776.1 +/- 720.5 mm^3. The MNNG (BAG) tumors also grew progressively, to a maximal volume of 420.3 +/- 234.9 mm^3. All mice survived until day 28, when euthanasia was necessary. At this time, primary tumors were harvested, grown in culture, and stained with X-Gal; all tumors stained negatively. See below (fig. 23).

FIGURE 22: Double Tumor Experiment - Group 3 MNNG (IL2) Secondary Tumor

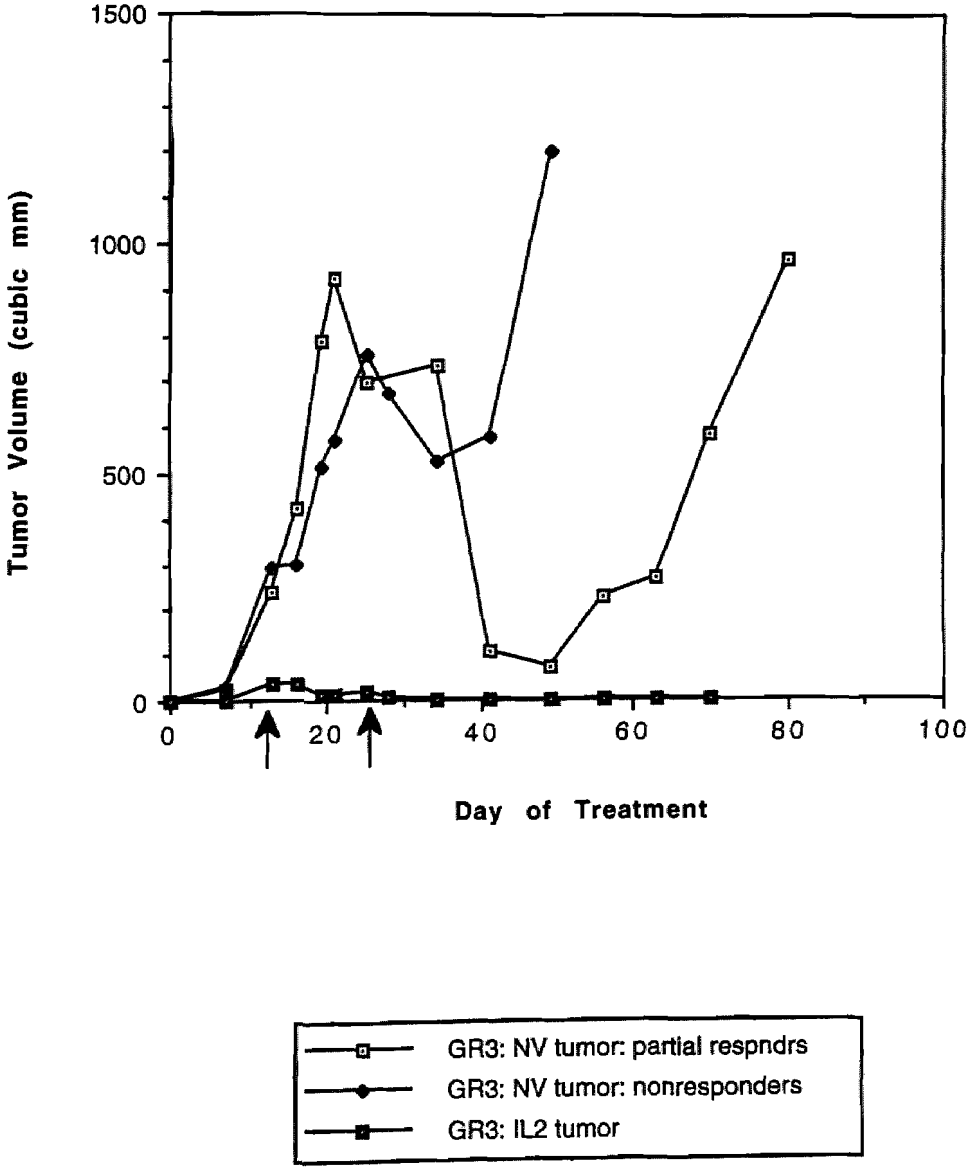
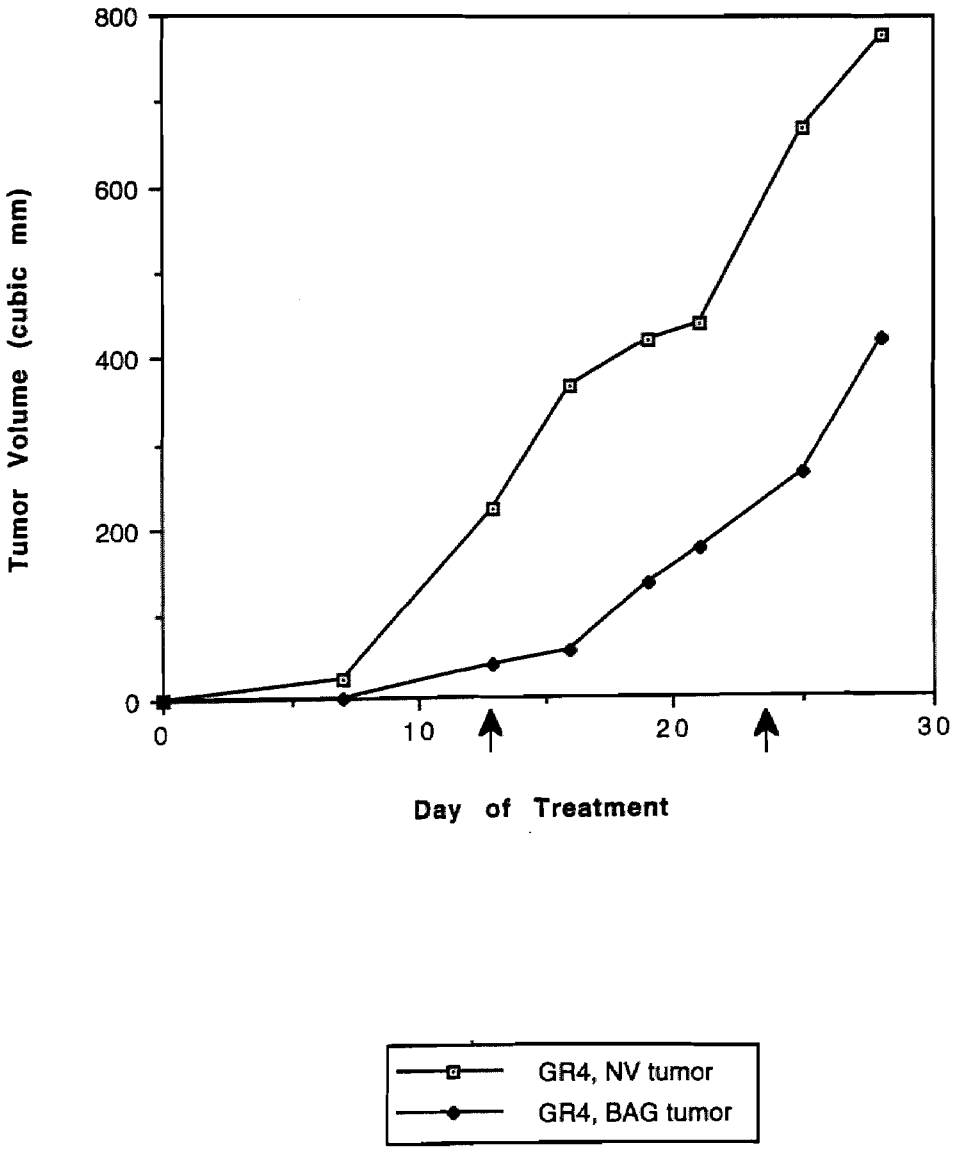


FIGURE 23: Double Tumor Experiment - Group 4
MNNG (BAG) Secondary Tumor



In Vivo Transduction Efficiency. Five subcutaneous MNNG tumors were injected with BAG vpc (described below under Treatment of Established Subcutaneous Osteosarcomas, Trial Two). Twenty-six days later, the mice were sacrificed and the tumors were harvested and grown in culture for two weeks. The percentage of wild type cells which were successfully transduced via retroviral vectors with the BAG gene was determined by staining harvested tumor cells with X-Gal. The results of X-Gal staining of these five tumors were variable. One tumor showed only 0.94% positively stained cells. In another tumor, the BAG gene was transferred more efficiently, at 17.08%. The average percentage of positive cells from these tumors was 6.64%. See table 15 below. Control tumors, injected with STK vpc, were completely negative in that X-Gal staining resulted in no color change of the cells (data not shown).

Table 15: *In vivo* Transduction Efficiency of the BAG Gene

	Number of Positive Cells	Percent
Tumor 1	45 / 564	7.98
Tumor 2	47 / 936	5.02
Tumor 3	174 / 1193	14.59
Tumor 4	8 / 861	0.93
Tumor 5	12 / 986	1.22
AVERAGE		**5.95**

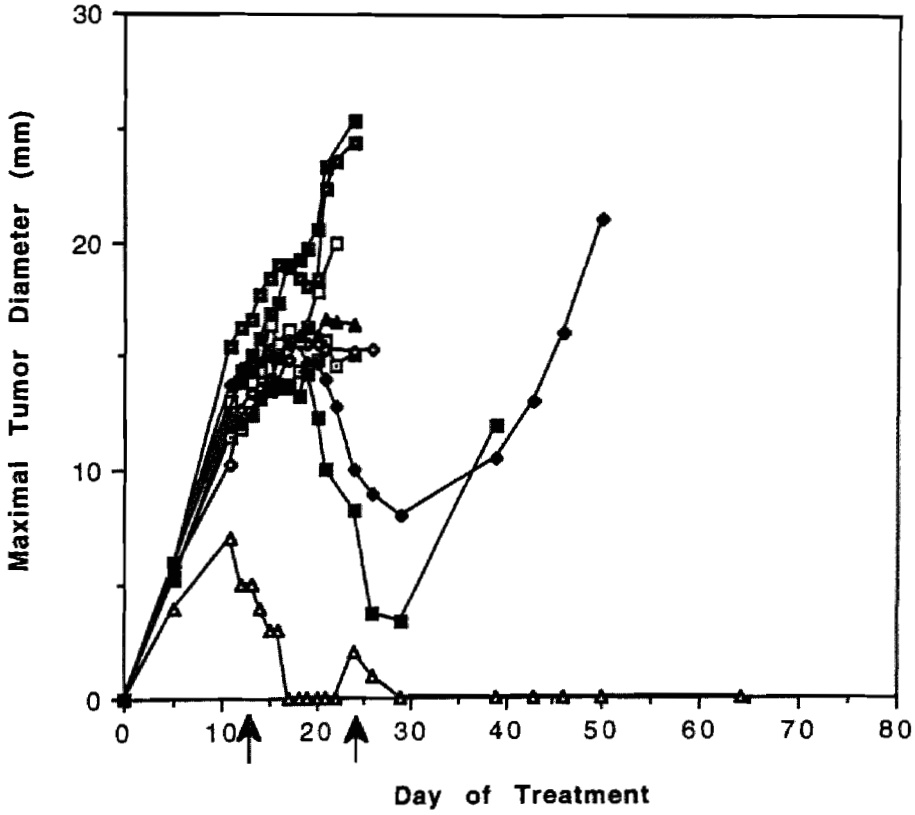
Treatment of Established Subcutaneous Osteosarcomas. (Fig. 24-30). Three separate trials were made to treat existing subcutaneous MNNG (NV) tumors by the injection of vector-producer cells. The results from each experiment are described below.

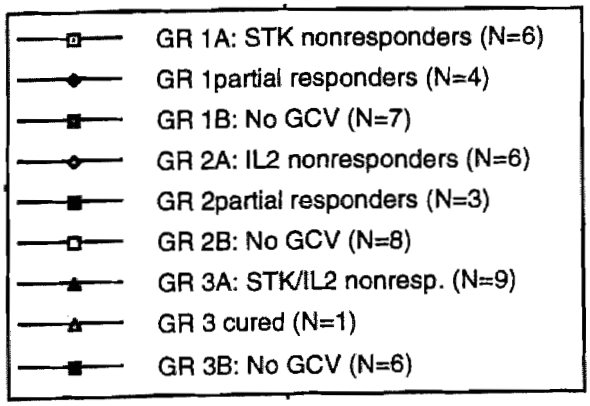
Trial One. Sixty-six nude mice were divided into four groups. On day one, MNNG cells ($5x10^6$) were introduced subcutaneously to the mid ventral surface. By day five, dense, nodular tumors had formed on all mice, with a maximal diameter of 5.73 +/- 0.20 mm. On day five, all mice received an injection of vpc (1.5×10^7) directly into the growing tumor mass. The 17 mice in Group 1 received STK vpc; the seventeen mice in Group 2 received IL2 vpc; the 16 mice in Group 3 received a mixture of STK and IL2 vpc ($7.5x10^6$ cells each, fig. 26); and the 16 mice in Group 4 received BAG vpc (fig. 27). At least half the mice in each group received GCV (3 mg in 1 ml HBSS) twice daily between days 13 and 24; the other mice received saline injections. The composite results are shown below (fig. 24). The number of responding mice (i.e., those experiencing at least a 50% reduction in tumor volume) in GCV-receiving groups are described in table 16 below.

Table 16: Treatment of Established MNNG Tumors with Vpc (Trial 1)

GROUP	Number of Responders	Percent	Cured
GR 1 (STK vpc)	4 / 10	40	0
GR 2 (IL2 vpc)	3 / 9	33	
GR 3 (STK and IL2 vpc)	1 / 10	10	1 (10%)
GR 4 (BAG vpc)	0 / 8	0	0

FIGURE 24: Treatment of Established Osteosarcomas with Vpc (Trial 1)



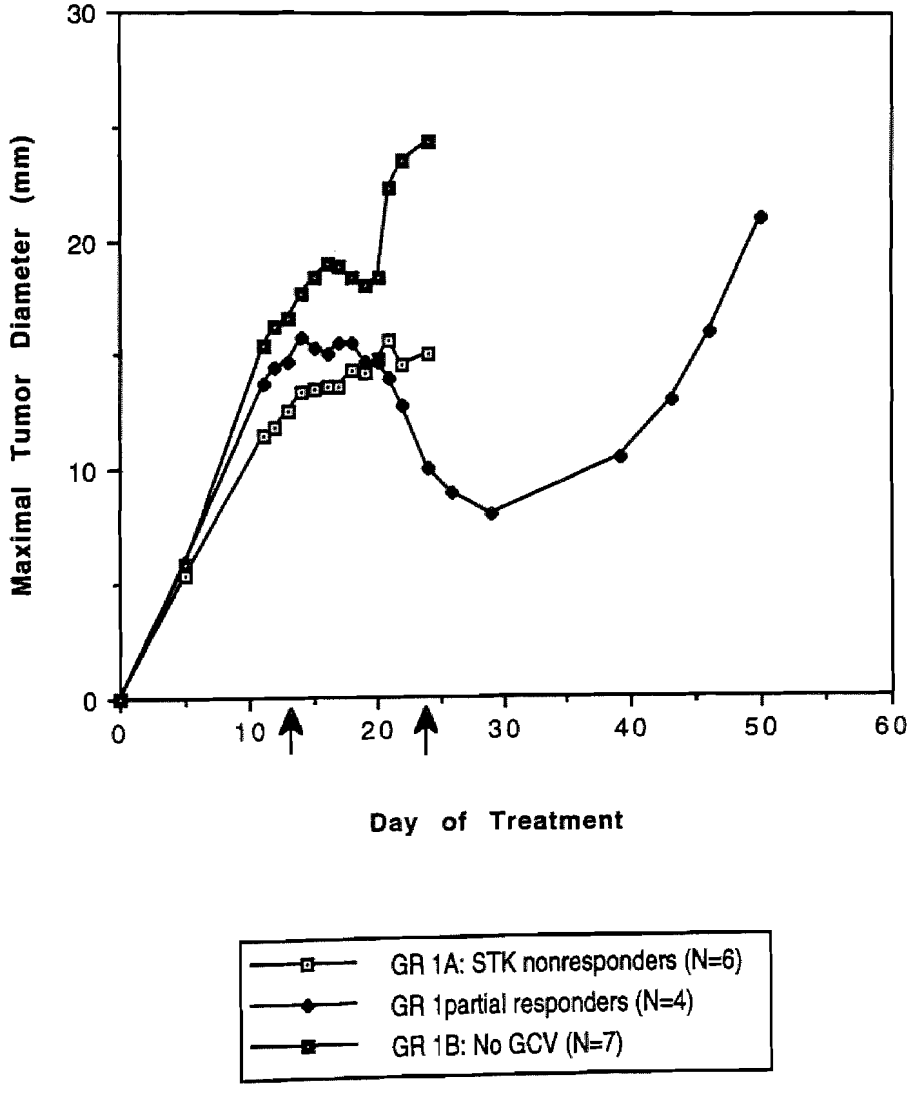


Four of ten mice (40%) in Group 1 (STK vpc) receiving GCV showed a partial response to therapy, experiencing at least a 50% reduction in maximal tumor diameter. By the first day of GCV injections (day 13), the maximal tumor diameter in among these mice was 14.75 +/- 2.0 mm. By the next day, the maximal diameter had increased to 15.75 +/- 2.0 mm; Midway through the GCV course (day 18), the tumors had not changed significantly in size (maximal diameter of 15.5 +/- 5.6 mm). Thereafter, the tumors began a gradual shrinkage process. By the final day of GCV treatment (day 24), the maximal tumor diameter had shrunk to 10.33 +/- 3.67 mm. The tumor diameters reached an average low at day 29, at 7.67 mm +/- 1.83 mm, or a reduction of 51.3% from the largest diameter. After this, the tumors began to expand once again, to a maximal diameter of 21.0 +/- 2.25 mm at day 50, when the mice were euthanized.

Six of the ten mice (60%) receiving GCV in Group 1 (STK vpc) did not show a significant response to therapy. At day 13, the average maximal tumor diameter was 12.5 +/- 2.46 mm. Midway through the GCV treatment course (day 18), the tumors had increased in diameter to an average of 14.17 +/- 4.11 mm. By day 24, the average maximal tumor diameter of these mice had grown to 15 +/- 5.49 mm, and the mice were euthanized. One of these mice had died of GCV toxicity on day 18.

Seven mice in Group 1 (STK vpc) received saline injections rather than GCV; these mice showed progressive tumor growth (day 13: maximal diameter = 16.57 +/- 1.29 mm; day 18: 18.71 +/- 1.98 mm; day 24: 24.71 +/- 4.97 mm) until euthanasia was necessary at day 24. The average tumor measurements for these mice were significantly larger (up to 39.3%) at corresponding days than those for the nonresponders in Group 1 who received GCV. See below (fig. 25).

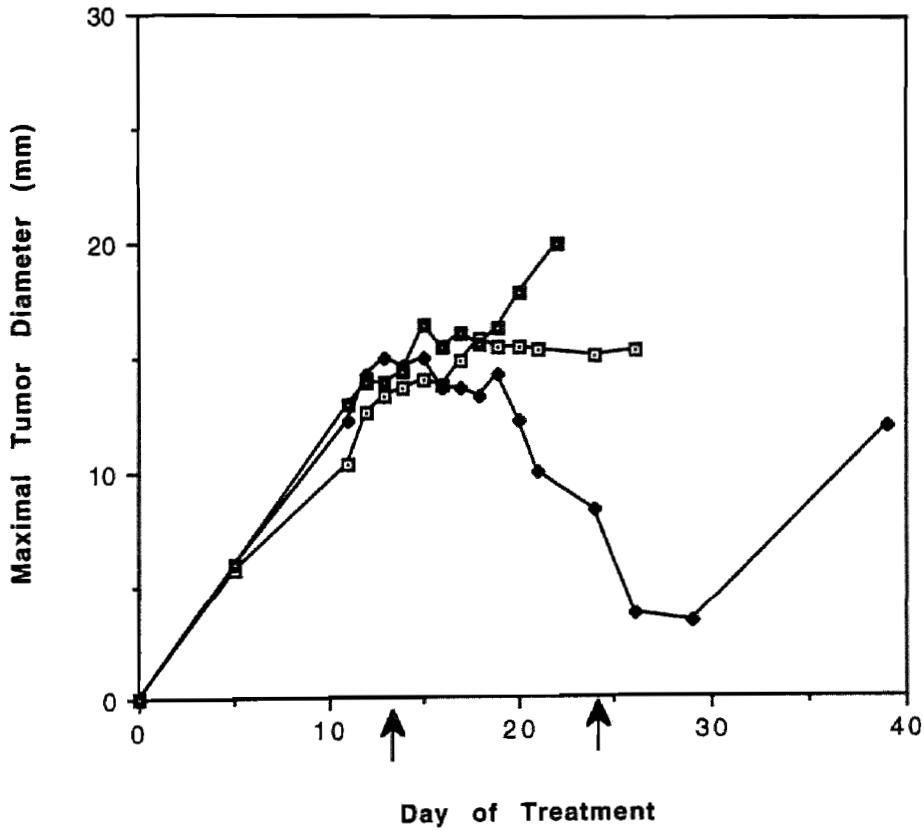
FIGURE 25: Treatment of Established Osteosarcomas with STK Vpc (Trial 1)



In Group 2 (IL2 vpc), nine mice received GCV and eight received saline. Three of the mice (33%) in the treatment group showed a significant reduction (an average of 77.8%) in tumor diameter. By day 13, these three mice had a maximal tumor diameter of 12.33 +/- 1.43 mm; midway through GCV treatment (day 18), the tumor diameters had increased slightly, at 13.33 +/- 4.5 mm. By the final day of GCV injections (day 24), the average maximal diameter had decreased significantly to 8.33 +/- 2.52 mm. The shrinkage trend continued until day 29, when the smallest maximal diameter (3.33 +/- 3.21 mm) was achieved. Thereafter, the tumors began to grow again, to a maximal diameter of 12.0 +/- 4.58 mm by day 39, when the mice were euthanized.

Six of the Group 2 mice receiving GCV (67%) showed no significant reduction in tumor size throughout the experiment. On day 13, the average maximal tumor diameter was 13.33 +/- 1.7 mm; these had expanded to 15.83 +/- 2.14 mm by day 18, and remained near this size until day 24 (15.178 +/- 3.9 mm) until these mice were euthanized. The eight mice in Group 2 which did not receive GCV showed progressive tumor growth which was not significantly different than the nonresponding mice who did receive GCV. On day 13, these control mice had an average tumor measurement of 13.88 +/- 1.13 mm; by day 18, these tumors had grown to 15.75 +/- 1.07 mm, and by day 24, growth had continued to an average maximal diameter of 20.63 +/- 3.68 mm. See below (fig. 26).

FIGURE 26: Treatment of Established Osteosarcomas with IL2 Vpc



In Group 3, mice received a mixture of STK and IL2 vpc. Nine of the ten mice (90%) in the GCV-positive subgroup showed no significant reduction in tumor size. By the first day of GCV treatment, the maximal tumor diameter was 10.89 +/- 1.1 mm; by day 18, the average diameter had increased to 15.89 +/- 2.05 mm; and by day 24, the average diameter had increased to 16.33 +/- 3.8 mm, and the mice were euthanized. One mouse (10%) in this group was cured of its tumor as a result of therapy. At day 11, the maximal diameter of this tumor was 7 mm, by day 17, no evidence of tumor remained. This mouse showed transient regrowth (2 mm) between days 24 and 26, but then remained tumor-free throughout the eight-week follow-up period. The six mice in Group 3 who received no GCV showed progressive tumor growth; on day 13, the average maximal tumor diameter of these mice was 14.0 +/- 1.32 mm. By day 18, the diameter had increased to 19.0 +/- 2.2 mm, and by day 24, the diameter peaked at 25.33 +/- 5.37 mm, at which point the animals were sacrificed. There was no significant difference in the growth patterns of the nonresponding GCV-positive mice and the control mice not receiving GCV. See below (fig 27).

In Group 4, mice received BAG vpc. The eight mice in this group who received GCV showed a slight (20.7%) shrinkage of maximal tumor volume. On day 13, the average tumor was 15.13 +/- 0.94 mm in diameter. Midway through the GCV course (day 18), the tumors were not significantly different in size, at 14.38 +/- 2.44 mm; it was on this day that the difference in tumor size between these mice and the controls not receiving GCV became statistically significant ($p < 0.05$). By the last day of GCV, the tumors averaged 12.88 +/- 4.46 mm in diameter. For the control mice, progressive growth was observed (14.63 +/- 2.32 mm at day 13; 18.88 +/- 1.63 mm at day 18; 26.43 +/- 3.2 mm at day 24). See below (fig. 28).

FIGURE 27: Treatment of Established Osteosarcomas with STK and IL2 VPCs (Trial 1)

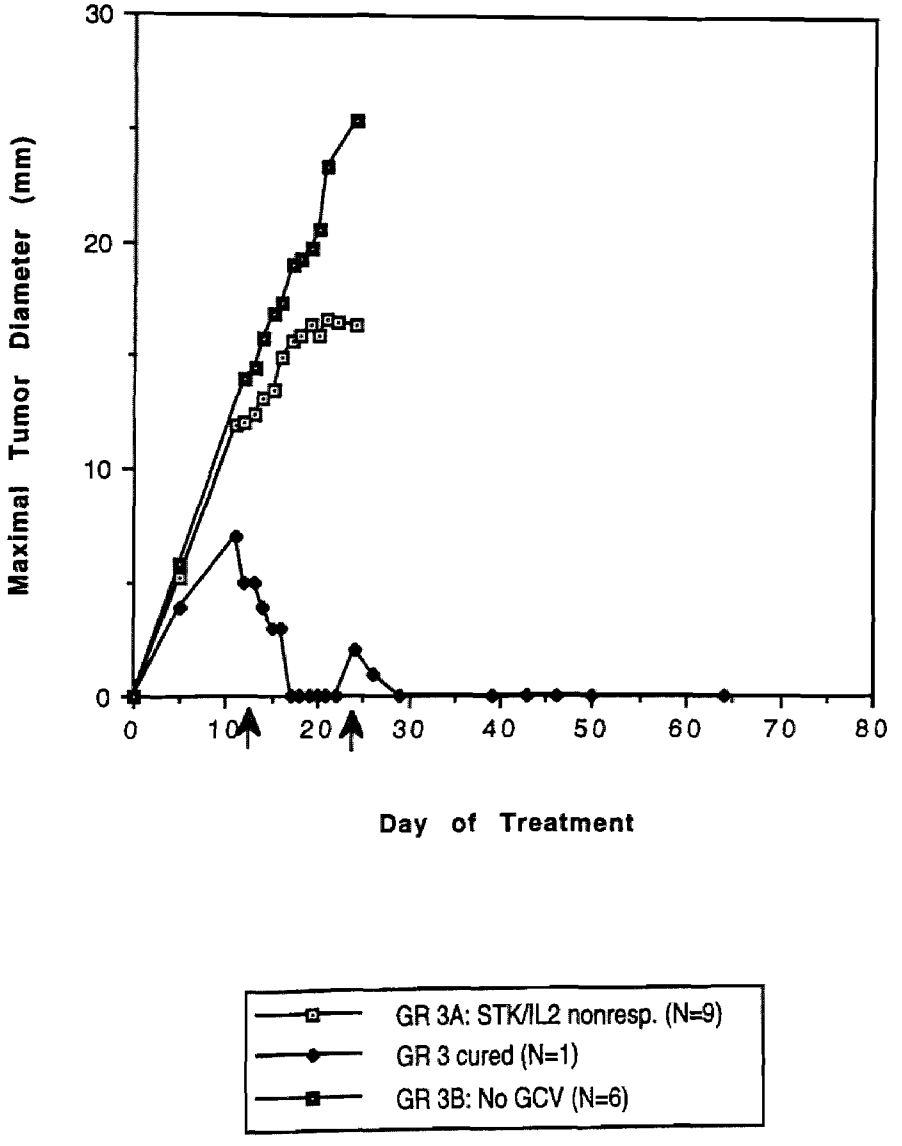
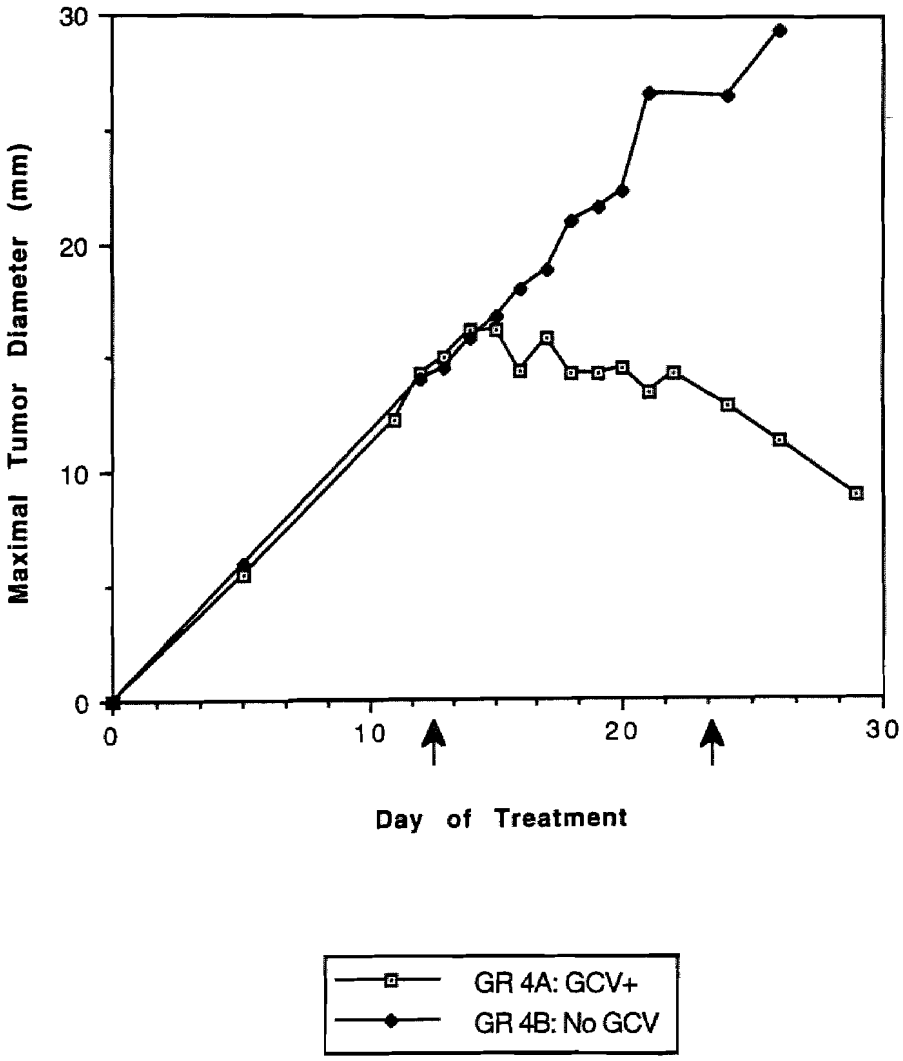


FIGURE 28: Treatment of Established Osteosarcomas with BAG Vpc (Trial 1)

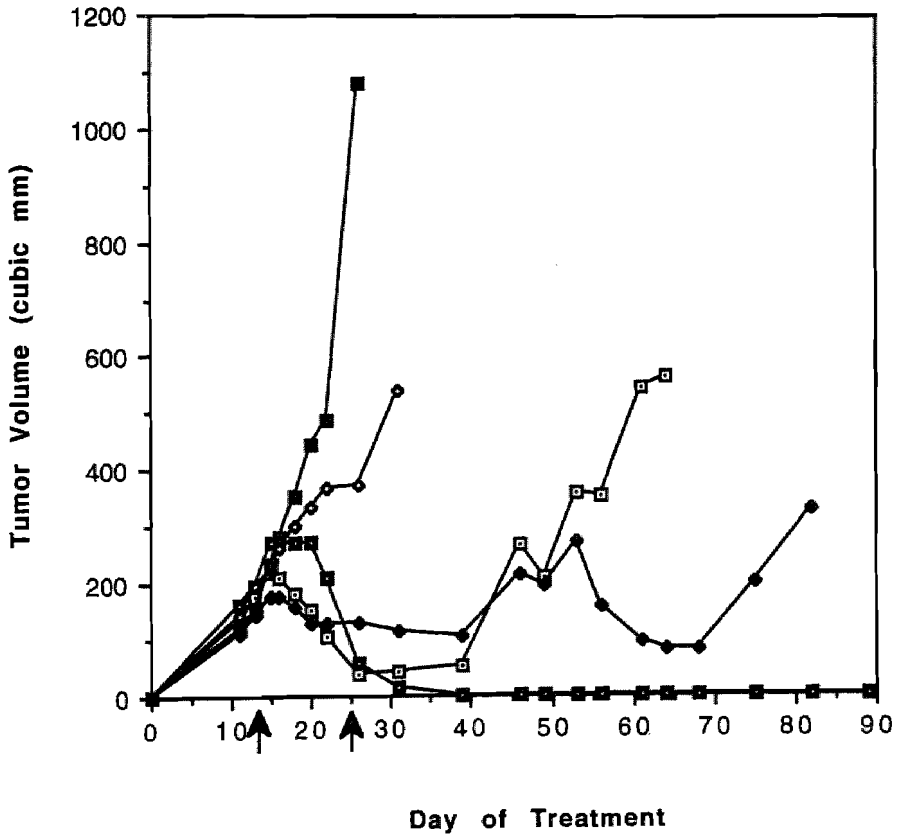


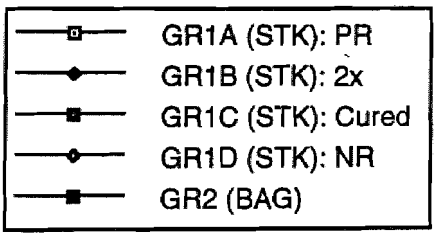
Trial Two. In this trial, thirty nude mice were divided into two groups. An initial inoculum of MNNG (NV) cells ($5x10^5$) was given to each of the thirty nude mice. By day 5, these tumors had grown to an average volume of 61.4 +/- 14.5 cubic mm. At this time, vector producer cells were injected intratumorally. Group 1A contained ten mice, which received a single dose of STK vpc ($2x10^7$). Group 1B contained ten mice which received one dose of STK vpc ($2x10^7$) and would receive a second dose of STK vpc two weeks later. Group 2A contained five mice which received a single dose of BAG vpc ($2x10^7$). Group 2B contained five mice which received one dose of BAG vpc and would receive a second dose of BAG vpc two weeks later. Table 17 below shows the number of mice per group showing at least a partial response (i.e., a 50% or greater reduction in tumor volume) to therapy. The overall results are depicted graphically below (fig. 29).

Table 17: Treatment of Established MNNG Tumors with Vpc (Trial 2)

GROUP	Number of Responders	Percent	Cured
GR 1A (Single STK vpc injection)	9 / 13	69	1 (8%)
GR 1B (Two STK vpc injections)	6 / 6	100	0
GR 2 (Single BAG vpc injection)	0 / 9	0	0

FIGURE 29: Treatment of Established Osteosarcomas with Vpc (Trial 2)





KEY:
GR1A = partial responders (N=8)
GR1B = two STK vpc treatments (N=6)
GR1C = cured mouse (N=1)
GR1D = nonresponders (N=4)
GR2 = controls (N=9)

Each of the twenty mice in Group 1 received STK vpc ($2x10^7$ cells). By day 13, these tumors had grown to an average volume of 171.6 +/- 23 cubic mm. GCV commenced at day 13, and continued for twelve days for those mice in Group 1A and one week for those in Group 1B. After a recovery period, mice in Group 1B received a second intratumoral injection of STK vpc on day 46, followed by a twelve day course of GCV from days 53-59. In Group 1A, two mice died from GCV toxicity. In Group 1B, eight mice were given a second STK vpc injection; six of these mice survived the process. The two other mice in Group 1B had highly vascular tumors and were excluded from a second vpc injection; they were regrouped into Group 1A and given GCV for an additional five days to complete the twelve day course. Surviving mice were followed until day 85.

One mouse in Group 1A was cured of its tumor. The tumor volume of this mouse peaked on day 15 (the third day of GCV) at 272.3 cubic mm; it remained this size until near the end of the GCV period. On day 22, the tumor had shrunk 23% to a volume of 209.44 cubic mm. Two days later, it had decreased in volume by another 72%, to 58.6 cubic mm. By day 39, just a residual inflammation remained, and no traces of any tumor remained by day 49. This mouse remained tumor-free for the next six weeks.

Eight mice of 13 in Group 1A (STK x1) showed a partial response to treatment. The average tumor volume of these mice peaked on day 15 at 221.0 +/- 50.6 cubic mm. During the course of GCV treatment, these tumors shrank progressively, to 183.9 +/- 75 cubic mm (16.8%) by day 18, to an average low of 35.9 +/- 22.4 cubic mm (an average total reduction of 83.8%) after the completion of GCV at day 26. Each of these mice then showed substantial regrowth until euthanasia was necessary.

Four mice in Group 1A (STK x1) showed no significant response. From an average volume of 198.4 +/- 63 cubic mm at day 13, the tumors nearly doubled in size to 369.1 +/- 62.9 cubic mm by day 22 at the end of GCV treatment. These animals were euthanized at day 26.

Each of the six mice in Group 1B (STK x2) showed a significant ($p< 0.05$) initial response to treatment. By day 13 (when primary GCV treatment was initiated), these mice had tumors with an average volume of 145.6 +/- 55.8 cubic mm. The tumors peaked in size on day 15, with an average volume of 177.3 +/- 67.6 cubic mm. Thereafter, the tumors began to decline in size; by day 20 (the final day in the initial GCV period) the tumors had shrunk by 25.9% to an average volume of 131.3 +/- 92 mm. This trend continued until day 31, when the tumors were at an average low with a volume

of 113.4 +/- 64.4 cubic mm. Three of the six mice began to experience regrowth at this point; the other three continued to show regression, to an average of 13.96 +/- 4.0 cubic mm at day 39; thereafter, these mice too began to show regrowth of the tumor. At day 46, a second STK vpc injection ($1.5x10^7$ cells) was given intratumorally. The tumors were highly variable in size at this point (10.47, 31.42, 36.65, 188.5, 263.9, and 769.7 cubic mm). By day 53, the tumors had grown by an average of 19.8% to 270.3 +/- 236 cubic mm; at this point, GCV was again commenced for a week. By day 56, each of the six tumors had begun to regress again, to an average of 155.5 +/- 143 mm, which was 42.5% smaller than three days earlier. Two of the six mice became sick and died on days 57 and 59. The four other mice continued to show regression. By day 61, the tumor volume average was 96.08 +/- 127 cubic mm, or 64.4% less than the previous high for these four mice. By day 68, these four tumors were at an average low volume of 79.3 +/- 78.6 cubic mm. After this, the tumors again began to grow, until it became necessary to euthanize the animals.

Group 2 contained ten mice, each of which received an injection of BAG vpc ($2x10^7$ cells) directly into the tumor on day 5. By day 13, the tumors had increased in size to an average volume of 152.2 +/- 40.5 cubic mm. Mice in Group 2A were given the standard 12-day GCV course, while those in Group 2B received GCV for one week only. In Group 2A, one mouse died midway through the GCV injection cycle. In Group 2B, three of three mice died after receiving a second BAG injection on day 31. At this time, the remaining five mice in Group 2 were euthanized and the tumors were harvested, cultured, and stained with X-Gal to measure the efficiency of gene transfer. See table 15 above.

Trial Three. Twenty-one nude mice were given a subcutaneous inoculum of MNNG (NV) cells ($2.5x10^5$) on day 1. By day seven, all mice had developed tumors with an average volume of 2.13 +/- 0.70 mm. At this time, STK vector producer cells ($1.5x10^7$) were injected as a single inoculum into the tumor mass. GCV was administered from day 13 to day 24. Two mice (9.5%) died from apparent GCV toxicity during the treatment course. The results of this experiment are summarized in Table 18 below, and depicted graphically in Figure 30.

Table 18: Treatment of Established MNNG Tumors with STK Vpc (Trial 3)

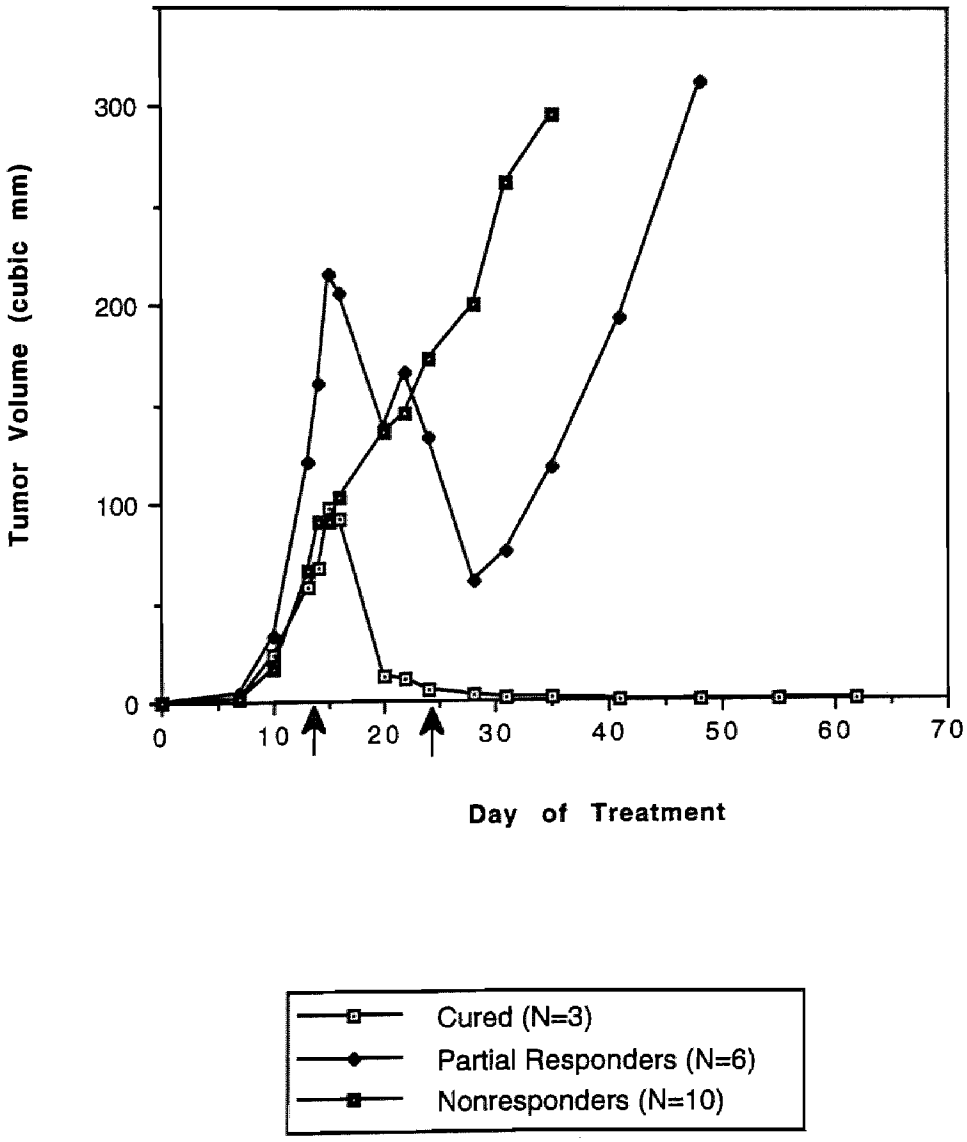
Number of Responders	Percent	Cured
9 / 19	47	3 (16%)

Three of the nineteen informative mice (15.8%) were cured of their tumors as a result of the genetic therapy. Two of these mice had tumors which remained relatively small (maximal volume of 15.71 cubic mm and 33.0 cubic mm on days 13 and 14 respectively); the other mouse developed a larger tumor, which peaked in size at 251.3 cubic mm on day 16. By the last day of GCV treatment, each mouse had only residual tumors (average volume 4.89 +/- 3.7 cubic mm). By day 41, the sites which had been tumorous were completely normal in appearance. These mice remained tumor-free over the next three weeks.

Six (31.6%) mice showed a partial response to therapy, experiencing at least a 70% reduction (average = 83%) in tumor volume. Two of these mice (10.5%) showed over a 90% reduction in tumor volume. On day ten, tumors of these mice had an average volume of 33.5 +/- 7.7 cubic mm. By the beginning of the first day of GCV treatment, the average volume had nearly quadrupled to 121 +/- 45.6 cubic mm. The average tumor volume peaked at day 15, on the third day of GCV treatment, at 215.5 +/- 78.7 cubic mm. Thereafter, the tumors began to shrink. On the final day of GCV treatment, the tumors had shrunk to 133.4 +/- 106.6 cubic mm, an average of 35% from the maximal average volume. The average minimal volume in this group was seen on day 28, when tumors had a volume of 60.3 +/- 26.1 cubic mm. Thereafter, the tumor volumes began to climb steadily, to a level above the pre-treatment high (average = 312.3 +/- 144.4 cubic mm) by day 48.

Ten mice (52.6%) showed no significant reduction in tumor volume throughout the treatment course. On day ten, the mice in this group had tumors which averaged 16.1 +/- 5 cubic mm in volume. By day 13, the tumors had more than quadrupled in size, to an average volume of 65.6 +/- 29 cubic mm. Between days 14 and 16, the tumors grew progressively but at a slower rate, to an average of 103.2 +/- 34.7 cubic mm. Thereafter, the tumors grew rapidly, to an average volume of 172.3 +/- 29.6 mm on day 24, to a maximum of 296.9 +/- 57.3 cubic mm, when the mice were euthanized. See below (fig. 30).

FIGURE 30: Treatment of Established Osteosarcomas with STK Vpc (Trial 3)



DISCUSSION

Cells

Four human osteosarcoma cell lines were obtained from American Type Culture Collection. The TE85 line originated in 1975 from an osteosarcoma of a 13-year old female patient.[153] This line was subsequently transformed *in vitro* by N-methyl-N'-nitro-N-nitrosoguanidine (0.01 μg/ml) to form the highly malignant MNNG cell line.[154] The c-*met* oncogene is reportedly activated in MNNG cells.[155] The MG63 line was originally derived in 1977 from an osteosarcoma of a 14-year old male; this cell line is noteworthy in that it is reported to produce high levels of fibroblastic interferon.[156] The G292 cell line originated from an osteosarcoma of a 9-year old female in 1976.[157]

The MLM line was a lung metastasis derived from an orthotopic implant of MNNG cells into the tibial tuberosity of a nude mouse in 1993 at the Human Gene Therapy Research Institute. Because the spontaneous development of lung tumors in nude mice is a very rare event,[158] it is a safe assessment that the lung tumor arose from MNNG cells which traveled through the blood from the tibia to the lung, undergoing small but critical changes en route. Moreover, examination of fixed MLM cells at metaphase indicates a non-murine origin, as many metacentric and submetacentric chromosomes are visible; murine chromosomes are acrocentric. Histological distinctions (as well as differences in the *in vitro* experiments discussed below) suggest that MLM is a novel cell line which differs subtly from the parental MNNG line. An analytical chromosome spread of the MNNG and MLM cells would allow a direct comparison as to the differences in ploidy and chromosome morphology.

Transduction and Selection

Selection for NeoR gene expression became evident between the third and fourth days of incubation in media containing G418 (one mg/ml). Selective pressure was maintained for at least two weeks. Transduction with STK and IL2 seemed to occur with a similar efficiency; by the third day of incubation in G418, perhaps 10% of the cells remained adherent and viable. Transduction with vectors containing the BAG gene was significantly less efficient; typically by the second to third day, perhaps 98% of the cells had died, indicating a low level of transfer of the neomycin gene. These results correlate with the reported titers generated by each vector producer cell line. Higher efficiency would be expected for supernants containing a greater number of viral particles.

Assays to demonstrate gene transfer

Successful transduction of osteosarcoma cells with the herpes simplex thymidine kinase gene of the STK vector producer cells was evaluated by a functional GCV susceptibility assay (fig. 1). While this assay provides a good means to determine the functionality of the transferred gene, it is not indicative of the precise levels of HS-tk produced by the cells, nor the number of copies of the gene introduced. This is a drawback in that the operative range of activity for a given concentration of the HS-tk enzyme is unknown. Ideally, a quantitative measure of the amount of enzyme produced daily by a given number of cells would be determined; however, there is currently no ELISA or other immunoassay available for this purpose. In each case, however, the insertion of the STK gene resulted in a marked enhancement of sensitivity to GCV.

An enzyme-linked immunosorbent assay was performed on all IL2-transduced cell lines (fig 2). Each line was found to produce antibody-recognizable IL2 at clinically relevant levels. Differences between the cell lines may be related to cellular division, multiplicity of infection, avidity of the transcriptional apparatus, or secretory capacity. The non-IL2 transduced lines which were tested showed no significant difference in absorbance compared to the negative control.

Transfer of the ß-galactosidase gene was confirmed on the basis of X-Gal histochemical staining (fig 3). The active, transferred enzyme cleaves the X-Gal substrate to a chromogenic compound. A spectrum in color change is observed in the cells, from deep blue to no color change. This test is a good measure of gene incorporation, as the intensity of color development is proportional to the amount of enzyme being produced. Four of the BAG-transduced osteosarcoma cell lines showed around 80% of cells as obviously positive for transfer of the gene. In the G292 (BAG) line, less than 50% of the G418-resistant cells stained positively. The BAG vector producer line showed 91% of cells as positive. In theory, each of these cell lines, barring genetic drift of the vector, should turn blue to indicate transfer of the BAG gene. Cells evaluated as negative may actually have been transduced, but are expressing only low levels of ß-galactosidase, so that the color change is not apparent with light microscopy. The differences may also relate to copy number. Also, there may be a range in membrane permeability of the X-Gal solution; cells absorbing relatively less of this chemical would be expected to stain less intensely.

Growth Assay (table 1). The purpose of this assay was to obtain an estimate for the *in vitro* growth rate of each original and modified cell line, and to determine whether retroviral-mediated transduction with the various genes would induce a significant effect on this growth rate. Furthermore, although *in vitro* and *in vivo* results cannot be expected to correlate exactly, an awareness of the proliferative rate is useful in accounting for trends observed in animal studies.

Placing the cells in order of generation time by transduction category within each cell line, the BAG-transduced cells rank first in overall proliferative rate. The non-transduced and STK-transduced cells rank next, having the same intermediate relative position. IL2-transduced cells expanded at a slower overall rate.

There are a number of possible explanations as to how retroviral gene transfer might affect generation time. Perhaps the most likely prospect relates to the fact that any given population of cells is polyclonal or oligoclonal, being comprised of several subpopulations which exhibit different molecular characteristics. The growth rate of these different subpopulations will be likely to range along a continuum. As retroviral infection is largely a random process, the small percentage of cells which survive selection may be considerably different from the bulk population, a scenario similar to "bottle-neck" evolution. Thus, happenstance may be the primary governing force behind the mean growth rate of the cells that are selected.

Several other factors may also affect the generation time. The site of integration into the genome of the target cell could have an impact. Integration near the promotor region or other regulatory sequences of genes involved in the cell cycle might also affect growth rate. Interruption of genes in this family by the introduced sequence could result in an increased growth rate (if an inhibitory gene were affected) or a slower growth rate (if a gene directing cell cycle progression were altered). Of course, retroviral integration into the genome of the target cell is a nearly random event, and interruption of any gene at all, particularly those regulating the cell cycle, is unlikely to occur on a regular basis. Altered growth patterns could result from an interaction with regulatory proteins (like repressors), by effecting a shift in the transcriptional profile of the cell, or by causing cells to increase the expression of certain genes involved in replication; cell-to-cell effects are also possible. These events could easily depend upon the conformational properties of the novel proteins, and might be amplified in multiply-transduced cells.

It must be mentioned that in most cases, the growth assay was performed only once for each cell line. Duplicate counts were done in each case, yet it would be

necessary to conduct this assay several more times to discern with confidence the subtle differences that may exist between the growth patterns of the various cell lines. However, definite trends have been identified. For example, each cell type from three osteosarcoma lines (MNNG, MLM, and TE85) all replicated at a similar, relatively rapid pace (about 20-30 hours), which may reflect their common origin. Also, the G292 cells grew significantly slower than any other cell type (which may contribute to the relatively erratic behavior of these cells in the other assays). This could indicate a fundamental difference which exists between osteosarcomas of disparate origin; also, G292 may have undergone alterations in various metabolic pathways which would necessitate the provision of some unidentified nutritional supplement for optimal growth. The trend for cells producing high levels of IL2 to grow at a more leisurely pace has been noted in previous studies, but most evidence appears to be anecdotal.

In actuality, the growth rate of a polyclonal population of cells is likely to be highly variable and dependent upon both extrinsic and intrinsic factors. Such factors might include passage number (indeed, significant variations might be expected to arise by random mutation in a population of cells maintained over the course of several weeks), density at previous passage, length of lag phase, nutritional considerations, and the secretion of growth stimulatory and inhibitory factors. Some of these variables do not lend themselves to experimental manipulation.

GCV-Sensitivity Assay (figs. 4-8).

Specific comments are made concerning results for each cell line. This is followed by general comments concerning this series of experiments.

MNNG. The MNNG (STK) line was highly susceptible to GCV. A 98.4% reduction in growth was seen at the lowest concentration of GCV, and the inhibition rose to 99.5% at the highest concentration ($p < 0.001$ for DPMs between no GCV and each concentration of GCV). The MNNG (NV) line also showed a susceptibility to GCV (19.2% decrease in proliferation at 2 μg/ml to a maximum of 61.9% at 10 μg/ml). Transduction of the MNNG line with the IL2 gene seemed to have a slight protective effect; only a small proportion (2.6%) of these cells died at a GCV concentration of 2 μg/ml. Even at the highest tested concentration of GCV, only a 36.5% reduction in proliferation was observed. However, the differences between the wild type and IL2-transduced line are not statistically significant due to the small sample size.

MLM. The MLM (STK) line was also highly susceptible to GCV. At the lowest concentration of GCV, a 99.1% decrease in proliferation was seen. At the highest concentration, the decrease in proliferation rose to 99.75% ($p < 0.001$ for DPMs between no GCV and each concentration of GCV). The unmodified MLM line demonstrated some susceptibility to GCV (44.1% decrease in proliferation at 2 μg/ml GCV up to 68.9% at 10 μg/ml); the differences in DPMs between the replicates without GCV and those with 10 μg/ml GCV are significant to the 95% confidence level. The susceptibility of the IL2-transduced line ranged from a 23% decrease in proliferation at 2 μg/ml GCV, up to 44.2% at 10 μg/ml, and the differences in DPMs between 0 and 10 μg/ml GCV are statistically significant to the 95% confidence level. The susceptibility of the IL2-transduced line is significantly ($p < 0.05$) less than that seen with the wild-type line.

TE85. Like its derivative strains discussed above, the parental TE85 line also showed high susceptibility to GCV when transduced with the STK vector. The decrease in proliferation ranged from 98.9% at 2 μg/ml to 99.4% at 10 μg/ml ($p < 0.001$ for DPMs between no GCV and each concentration of GCV). The unmodified line was somewhat less susceptible to GCV than those above (ranging from 17.4% at 2 μg/ml to 46.6% at 10 μg/ml). The IL2-transduced cells were inhibited at a slightly higher level than the non-transduced cells (27% at 2 μg/ml, up to 44.2% at 10 μg/ml).

MG63. The STK-transduced cells of this osteosarcoma showed high susceptibility to GCV (99.1%) at the lowest concentration of GCV. The reduction in proliferation increased directly with GCV concentration (99.7% at 10 μg/ml; $p < 0.001$ for DPMs between no GCV and each concentration of GCV; $p < 0.05$ between DPMs for 2 and 10 μg/ml and between DPMs for 5 and 10 μg/ml GCV). The wild type cells showed a marginal decrease in growth with GCV (17.4% at 2 μg/ml to 45.9% at 10 μg/ml). The IL2-transduced cells proved to be more prone to GCV-mediated demise than did the non-transduced cells; the reduction in MG63 (IL2) cells ranged from 34.1% at 2 μg/ml to 63.4% at 10 μg/ml ($p < 0.05$ between DPMs for 0 and 10 μg/ml). These cells were also the most sensitive to GCV of any IL2-transduced line.

G292. This cell line showed lower levels of GCV-susceptibility compared to the other osteosarcomas. The STK-transduced cells showed a relatively low (87.7% to

93.7%) sensitivity to GCV ($p < 0.05$ for DPMs between no GCV and each concentration of GCV). The wild type cells showed a low level of inhibition in GCV (21.5% to 35.3%). The IL2-transduced G292 cells showed a modestly better survival pattern than did the unmodified cells (7.9% decrease in proliferation at 2 μg/ml to 27.1% at 10 μg/ml), indicating that the IL2-transduced cells were slightly protected from GCV-mediated inhibition.

All STK-transduced lines showed statistically significant susceptibility to GCV. All HS-tk positive cells should be inhibited in the presence of GCV. Here, the highest rate achieved was 99.75%. There are a number of possible reasons which might explain this minor discrepancy. In these experiments, cells were incubated with GCV for 48 hours. Only cells undergoing DNA replication in this time span would be targeted. The fraction of remaining cells could represent a slow-growing subpopulation; a meandering replication rate would decrease the opportunity for incorporation of GCV-triphosphate into a growing DNA strand, and could prolong the survival of susceptible cells. This explanation is particularly likely to account for the substantially lower susceptibility seen in the sluggish G292 line; with an average generation time in excess of 80 hours, perhaps only about half of the cells underwent replication during the incubation with GCV (and some of the cell death might be accountable for by the bystander effect). Moreover, it has also been reported that GCV is catabolized slowly by cells,[85] it is possible that a longer incubation period would have induced greater inhibition.

Furthermore, the radiolabel could have been expressed by dying cells that had not yet lost their adherence properties. In other words, greater inhibition may have been achieved, but not realized in the time allowed for analysis. The highest level used here is lower than the minimal serum level necessary for *in vivo* activity.[85] Some cell types may be better equipped to recognize and sequester low levels of foreign substances. Higher efficiency has been demonstrated by increasing the GCV concentration (data not shown).

Another possibility for the less-than-complete inhibition of STK-transduced cells is that a small fraction of the cells were GCV-resistant. This could result from mutations in the HS-tk construct in a small number of cells, followed by rapid expansion of this subpopulation during selective pressure. Indeed, it is conceivable that this type of event occurs infrequently though regularly in vector producer lines. For example, random point mutations in the vector could render the tk-promotor ineffective or knock out a critical codon which would impair the function of the resulting protein; retention of the neomycin

resistance and packaging sequences would allow this null vector to become incorporated into target cells. This mechanism could also be invoked to explain some of the functional variability observed with other vectors. GCV-resistance could also be a newly acquired trait stumbled upon by the molecular adaptability and good fortune of an occasional cell.

A limited susceptibility (up to 68.4%) was seen by HS-tk negative cells including each wild-type and IL2-transduced osteosarcoma. This is somewhat unexpected, in that these cells have not been engineered to express the gene for which GCV demonstrates preference. A possible explanation of this is low-level phosphorylation of GCV by the endogenous tk gene or other cellular kinases. The selectivity of GCV for the herpes-derived enzyme has been reported to be twenty-fold greater than for cellular enzymes,[85] which would support this explanation. In fact, the cytomegalovirus has no tk gene but is destroyed by GCV due to other cellular kinases that produce GCV-TP.[159] Alternatively, GCV may induce a dose-dependent cellular toxicity that is unrelated to replicational interference. Previous work has shown that cell toxicity occurs in both HS-tk modified and unmodified tumor cell lines, and this toxicity increases with duration and concentration of GCV exposure.[160]

When incubated in GCV, these two of the five cell lines (MLM and G292) showed a slightly decreased rate of inhibition of the IL2 cells versus the wild type cells. This trend was identified for each concentration of GCV tested, and the effect was generally diminished as the GCV concentration increased. Because the figures have been standardized, any difference in total proliferative rate is nullified. Thus, all else being equal, this protective effect is most likely attributable to IL2 production. Because this data was generated *in vitro*, the cells were physiologically isolated from the immune system. As IL2 is classically considered to be a potentiating rather than a participatory molecule, this observation about IL2-transduced cells is somewhat novel. The IL2-protective effect could be either intracellular or intercellular, specific or non-specific, and may be inducible in some cell lines upon challenge. Possible mechanisms for the protective influence of IL2 could include a direct interaction with GCV or GCV-TP, secondary induction of GCV-resistance in selected populations, inhibition of cellular kinases, increased regulation of replication, or a generalized slowing of cellular replicatory processes. Alternately, receptor-bound IL2 could trigger a cascade of events which influences GCV uptake. Of course, this effect was seen in only two of the five cell lines, and may or may not be represent a general phenomenon of tumor cells.

Except as noted, the differences in proliferation between the varying concentrations of GCV for no-vector lines are not statistically significant to the 95% confidence level; the same is true for the IL2-transduced lines. Thus, it cannot be stated that the differences observed did not arise from chance or high oligoclonal selection in G418. This limitation can be attributed to the low sample size (i.e., triplicate), as well as the inherent variability in the experimental method. For example, in each well, the addition of a mere ten thousand cells was the goal, and each population of cells might be expected to incorporate the radiolabel at slightly different rates; the error is compounded by the fact that successive readings of the same filter mat can typically yield results which vary up to five percent. However, the trends discussed above are strong and uniform. It is likely that these trends would be statistically supported using a greater number of replicates.

***In Vitro* Bystander Assay** (figs. 9-13).

In the absence of a bystander factor, it would be expected that only cells transduced with the STK gene would killed upon incubation with GCV. Thus, coincubation of MNNG (NV) and MNNG (STK) cells with GCV should result in a decrease in proliferation proportional to the number of MNNG (STK) cells present. For example, in a mixture having 8% MNNG (STK) and 92% MNNG (NV), only 8% of the cells would be expected to die. When this assay is performed, however, a 95.1% decrease in proliferation is observed, indicating that the vast majority of unmodified tumor cells are killed along with those which are STK-transduced. Because these transduced tumor cells are not packaging viral particles, the conferral of sensitivity to GCV must be mediated through a derived factor.

Thus far, the identity of the bystander factor remains unknown. The leading hypotheses were presented in the introductory section above, and will be addressed further below. Regardless of the composition of the factor, the effect it mediates could have a profound impact on the applicability of gene therapy in the treatment of cancer. It would be very relevant in clinical trials to have an awareness of the strength of the bystander effect, and how much the factor might be diluted before losing its antitumor activity. This *in vitro* assay demonstrates both the presence and the potency of the bystander factor. Here, a strong suppressive effect (86.2% decrease) is seen in a mixture containing only 2% MNNG (STK) cells. Even as low as one percent MNNG (STK) cell, a 46.6% decrease in proliferation is seen. This suggests that delivery of the STK vector

to all cancer cells is not necessary to induce a significant response. The ability of the bystander factor to operate over a distance would have profound implications as a systemic therapy; this potential was explored in nude mice as described below.

The principle of this *in vitro* assay was to explore the potential of STK-transduced osteosarcoma cells to communicate a GCV susceptibility to wild type cells. The potential for IL2-transduced cells to enhance the bystander inhibit was also tested by co-incubating these cells with STK-transduced cells and wild type cells. In the initial five assays, IL2-transduced cells were also used to target wild type cells without STK-transduced cells, and as a control, STK-transduced cells were used to target IL2-transduced cells. A more stringent bystander assay was performed for the MNNG line; this assay tested only the effect of MNNG (STK) cells and a mixture of MNNG (STK) and MNNG (IL2) cells upon the proliferation of MNNG (NV) cells.

For each cell line, a decreasing percentage of STK-transduced cells resulted in an increased DPM. Thus, the bystander effect is greater with increasing concentration of STK-transduced cells. In each case, substitution of half of the STK-transduced cells with IL2-transduced cells resulted in a lessened bystander effect, indicating that this phenomenon is not improved by the presence of IL2 for these cell lines.

The data obtained from these five bystander assays must be interpreted with the following considerations. In each case, the assay was performed in triplicate. Due both to the closeness in value of the figures and the small sample size, the differences between three of the four experimental groups (i.e., STK : NV, STK/IL2 : NV, and STK : IL2) are not statistically significant at a given cellular ratio for each cell line. Likewise, because the values are so close to each other, the mixtures at the low end of transduced cell content (i.e., 1%, 2%, 5%, and 10%) are not significantly different from one another. Indeed, a ratio of 1% represents only 100 cells, which were transferred in a minuscule volume of 2 μl; the technical aspects at this order of magnitude make it difficult not to introduce a level of error. Furthermore, because the assays were performed at different times with different stock solutions of tritium, it is not possible to directly compare the results between cell lines.

For each cell line, however, the differences in DPM between 50% mixtures and the mixtures 10% and below are statistically significant ($p < 0.05$). The trends seen in the mixtures with the lower percentages of transduced cells are similar for each line, which infers a commonality. When the entire body of evidence is considered, a trend is firmly suggested. Moreover, when the bystander effect in the MNNG line was studied more

rigorously (using a four-fold greater sample size in each case), the results obtained were comparable, and were statistically significant to the 95% confidence level. This lends further credence to the results of the other bystander assays.

In some cell lines, the bystander effect appears to be enhanced by the presence of tumor cells which have been transduced with the IL2 gene. To investigate this possibility, MNNG (IL2) cells were coincubated with MNNG (STK) and wild type cells in another series of experiments (table 9). There are two treatment philosophies from which these data may be approached. In the first, a comparison is made between the proliferative effects in the presence and absence of MNNG (IL2) cells at a given percentage of STK-transduced cells. This comparison is directed at the mechanism of STK-mediated inhibition and the determination of whether this mechanism is enhanced through the presence of IL2-producing cells. In the second, the number of wild type cells is kept constant, and the effects of a given percentage of transduced cells (whether MNNG (STK) alone or an equal mixture of MNNG (STK) and MNNG (IL2) cells) are evaluated. This approach is perhaps more practical. In a clinical setting, the number of transduced cells to be administered must necessarily be limited to a finite number; in this context, then, the ratio of tumor cells and transduced cells is fixed. Thus, it is important to determine whether the best response would be achieved using only STK-transduced cells, or a mixture of both STK and IL2-transduced cells.

In these experiments, no synergy was seen between STK and IL2 transduced cells. At any given concentration of MNNG (STK) cells, there was no significant difference in the overall decrease in proliferation whether MNNG (IL2) cells were present or absent. Thus, MNNG (IL2) cells, included in exchange for a like number of MNNG (NV) cells, did not cooperate with MNNG (STK) cells to induce a larger response. A direct comparison indicates that in five of the six mixtures, the inclusion of MNNG (IL2) cells actually resulted in a slightly greater survival, which may indicate that MNNG (IL2) cells are a less avid target than are the wild type cells. Furthermore, at a given ratio of MNNG (NV) cells, a significantly greater response was achieved by MNNG (STK) cells alone rather than by a mixture of MNNG (STK) and MNNG (IL2) cells. Thus, the presence of MNNG (IL2) cells detracts from the bystander effect *in vitro*. However, these assays do not rule out an *in vivo* synergy between HS-tk and IL2, or a synergy *in vitro* in other tumor cell lines.

Establishment of *In Vivo* Tumor Models

As a prelude to animal studies, the first task was to determine which of the four original cell lines (MNNG, TE85, MG63, G292) would produce growth in nude mice. Of these four lines, only MNNG proved to be tumorigenic. This is a human osteosarcoma line (TE85) which was transformed *in vitro* with a chemical mutagen (N-methyl-N-nitro-N-nitrosoguanidine) to produce a highly malignant cell line. At both subcutaneous doses, large masses were evident within one week; these tumors grew rapidly until reaching a maximal allowable size (2x2x1 cm) by three weeks, at which time the mice were euthanized. In the tail-vein groups, all mice receiving MNNG cells developed subcutaneous tumors at the base of the tail; upon reaching critical tumor mass (3-6 weeks), the mice were sacrificed and the internal organs surveyed for metastatic growth. This pilot experiment was also informative in that no mice were observed to show any detrimental systemic response to the xenografted cells, and in no case did the cancer invade beyond the subcutaneous layer. The characteristic of this cell line to remain localized when introduced subcutaneously allowed it to be exploited as a model for further experiments which are described below.

Mice receiving implants of each of the other three cell lines (TE85, MG63, G292) failed to develop tumors. In the subcutaneous groups, small nodules developed at the injection site, at a size proportionate to the cell dose. The nodules were typically cleared within a week, and in no case did the nodules expand to a sustained tumor. No tumor growth was seen in any of the tail-vein groups. All mice were euthanized after six weeks; nothing unusual was noted at autopsy.

Failure of these cells to produce tumors is likely to be related to a combination of the following factors: slow growth rate, recognition and destruction by host defenses (including natural killer cells), or confrontation by an inhospitable environment (lacking essential nutrients). Spontaneous bone tumors are rare in laboratory mice.[161] In a survey of 19, 875 mice kept under standard conditions for 18 months, only six osteosarcomas were found.[6] This indicates that the incidence such tumors is less than 0.1%, and lends credibility to the assumption that the tumors that did develop were induced as a result of tumor cell implantation.

Safety

The comprehensive picture provided by the *in vivo* experiments discussed below contributes to the large body of evidence that the administration of vpc may be done

safely and without noticeable side-effects. Proper delivery of these murine fibroblast lines, injected either intratumorally or as mixtures with tumor cells, appeared to induce no untoward local or systemic reaction. Moreover, no differential in safety was seen between the STK, IL2, and BAG vpc (all modified mouse cell lines); all were equally well-tolerated. Control mice receiving separate subcutaneous inocula ($1x10^6$) of these vpc showed only transient induration at the injection site, which suggests that these cells are not tumorigenic. Subsequent examination at the implant site showed no gross injury, and histological evaluation of the implant site revealed normal tissue.

No mouse appeared to experience any ill-effects from the presence of retrovirus. This indicates that integration into normal tissue either did not occur or was not at all deleterious. No evidence of disease resulting from recombination to replication-competent virus was observed.

In vitro transduction of tumor cells with retroviral vectors followed by implantation of the altered cells had no added ill-effect upon the animals compared to implantation of unmodified tumor cells. Transduction with the IL2 gene actually was found to substantially decrease the tumorigenicity of the cells. This supports the tenet that tumor cells may be safely manipulated *in vitro*.

The adverse response by some mice during the therapeutic course is most likely attributable to GCV toxicity. These mice displayed what came to be recognized as a "classic" syndrome involving anergy, weight loss, posterior hunching, lethargy, flaking skin, and dehydration. Such symptoms are similar to those which have been identified as a toxic response GCV and are identical those seen in control mice which are given GCV at the same dose and duration (data not shown). These symptoms were most often seen during the second half of the twelve day GCV regimen, and typically persisted two to three days until death of the mouse. Some adjustments were made in subsequent experiments to minimize this morbidity by lessening the dose of GCV and isolating affected mice. However, affected mice seldom showed improvement, suggesting that serious pathology to the organ systems may develop in affected animals prior to any obvious outward sign. Previous work indicates that the concentration and duration of GCV treatment used here are the minimum necessary to produce a strong therapeutic effect (data not shown). GCV-related mortality remained between five and ten percent throughout this study.

Cellular Threshold of MNNG Tumorigenicity (fig 10).

Of the thousands of tumor cell lines with which investigators work, each type has unique *in vitro* and *in vivo* growth characteristics. Cell lines differ in species of tropism; for example, tumor cells derived from a given strain of mice typically will not grow in other strains. However, many tumors from other species will grow within the immunologically hospitable environs of the athymic mice. Cell lines also differ in organs of tropism. Cells from a similar line often show different patterns of growth when introduced to various organs within the same animal model. For example, it has been reported that a count of $4x10^4$ human osteosarcoma (KRIB) cells are sufficient to produce a tumor when injected intraosseously;[162] over twice this number of MNNG cells are necessary to induce a subcutaneous tumor. Other lines may only grow at a limited number of sites. Finally, tumor lines may differ drastically in the number of cells required to produce tumors. To illustrate this, a rat mammary carcinoma (MATB) will grow progressively and kill experimental animals within three weeks when as few as $1x10^3$ cells are introduced intraperitoneally,[163] while other lines are not tumorigenic at any practical dose.

Progressive growth in nude mice of xenogeneic tumors is influenced by tumor-related properties, such as the origin and type of tumor, and on the route of inoculation.[145] It is reported that human melanomas, sarcomas, and soft-tissue carcinomas can be transplanted successfully to nude mice, while other carcinomas (breast, prostate, stomach) are more difficult to establish.[164] Once ensconced, the growth rate of tumors may depend largely upon the ratio of proliferating cells to dead or dying cells. Tumor growth is also dependent upon mouse-related factors, such as the age, strain, and state of health.[165] Additional factors influencing tumor growth are extracellular matrix production, edema, bleeding, and necrosis.[166] Furthermore, within a human tumor transplant, only the neoplastic cells are of human origin; the connective and vascular tissue are derived from the mouse host.[167] A component of the variability in tumor growth may depend upon the extent of recruitment by individual tumors.

This variation in experience with different tumors was the rationale behind studying the cellular threshold required for MNNG cells to form tumors subcutaneously. Injection of $5x10^4$ cells resulted in no tumor growth; twice this number resulted in tumor growth in some (17.6%) mice. When at least $2x10^5$ MNNG cells are injected subcutaneously, all mice, without exception, develop tumors which have the capacity for progressive growth. This trend approximates the classical sigmoid curve and plateau

expected for such studies. From this data, and assuming a linear relationship between cell number and tumor development, the TD_{50} (cell number required for tumor development in 50% of animals) would be approximately 1.61×10^5 MNNG cells. Knowledge of the *in vivo* growth threshold was instructive in designing subsequent treatment protocols.

Mixtures of MNNG and STK vpc (fig 15).

This experiment was designed to determine whether retroviral-vector mediated gene therapy could, under optimal conditions, result in ablation of subcutaneous MNNG. The treatment was idealized both in the interval of vpc introduction and in the equal distribution of vpc among target cells. Mice in each of the four experimental groups received 1×10^6 MNNG (NV) tumor cells mixed with five times as many, twice as many, equal numbers of, or half as many STK vpc. Over the next seven days after implant, the vpc produced a continual supply of the HS-tk vectors. These retroviruses served as the vehicle for *in vivo* transduction, whereby the tk gene was insinuated into the DNA of neighboring MNNG cells. Through the existing transcriptional and translational machinery, these tumor cells now began producing the enzyme that would make them susceptible to elimination by GCV.

Mice in Group 1 received a total inoculum of 3×10^6 cells subcutaneously, at a 5:1 ratio of STK vpc to MNNG tumor cells. Immediately prior to the first GCV injection on day 11, all animals had developed spherical tumors with a maximal diameter of 7.4 +/- 2.9 mm. The first significant decrease in tumor size was noted three days later, when the average maximal tumor diameter had dropped to 6.2 +/- 1.6 mm. Thereafter, the tumors shrank rapidly. By the final day of GCV, the tumors had resolved to a maximal diameter of 1.75 +/- 2.75 mm, and were flat and scarlike. Two mice died during GCV treatment (on days 19 and 23). One of these mice had no residual tumor by day 20; the other three were cured by day 34 (2) and 49. Surviving mice were monitored through day 85 (at least six weeks after tumors were reported to be fully absent.) Mice receiving this ratio of cells and no GCV experienced progressive tumor growth.

Mice in Group 2 received a total inoculum of 1.5×10^6 cells (half the number of Group 1), at a 2:1 ratio of STK vpc to MNNG cells. By day 11, the tumors had grown to a maximal diameter of 8.6 +/- 3.68 mm. The tumor regressed at a similar pace to those in group 1. Two mice showed no evidence of tumor near the end of GCV (day 20 and 22). The other three mice retained small but measurable scars until day 64 and 72 (2). During

the two week follow-up until day 85, these animals remained healthy and tumor-free. Mice receiving this ratio of cells but no GCV experienced progressive tumor growth.

In Group 3, mice received a total inoculum of $1x10^6$ cells, at an equal ratio of STK vpc to MNNG cells. By day 11, the average maximal tumor diameter was 8.8 +/- 3.6 mm. These tumors also declined in size rapidly; all residual traces had disappeared from individual mice by day 22, 37, 57, and 75 (2). No regrowth was noted. Mice receiving this ratio of cells but no GCV experienced progressive tumor growth.

Mice in Group 4 received a total inoculum of $7.5x10^5$ cells, of which 67% were MNNG tumor cells. By day 11, the average maximal tumor diameter was 7.2 +/- 3.3 mm. These mice required a longer period of time to become tumor free. One mouse died during GCV injections (day 21); one mouse showed no evidence of tumor by day 57, and another by day 75. The other two mice retained small, non-neoplastic scars until the experiment was terminated at day 85. Control mice receiving this ratio of cells but no GCV experienced progressive tumor growth, to a maximal diameter of 20.33 +/- 3.8 mm by day 28.

Between each of the groups, no significant difference was seen between maximal tumor size by the first day of GCV treatment, despite the over six-fold range in initial tumor burden. Furthermore, no significant differences were seen between the maximal tumor diameters observed in any of the control animals. Because the difference between the groups was the number of STK vector producer cells, it may be inferred that these murine cells are less tumorigenic than are the malignant human osteosarcoma cells. The rate of tumor killing and clearance also did not differ significantly between the treatment groups. Tumors were consistently ablated with a 1:1 ratio of MNNG cells to STK vpc; the retention of minute scar-like patches in two mice in group 4 makes the smaller ratio somewhat less reliable. No mice showed regrowth during the follow-up period.

Mixing tumor cells with therapeutic cells prior to tumor implant does not approximate a treatment scenario; indeed, the glory of success here is analogous to that of a construction crew demolishing a building made of explosive bricks. However, it was imperative to ascertain whether these tumor cells would be at all amenable to our gene therapy strategy. In some cases, tumor cell lines have been sufficiently belligerent to grow progressively despite co-inoculation with vector-producing cells.[168] Here, one million MNNG cells were eliminated with as few as $5x10^5$ therapeutic cells. At this ratio, it is likely that less than 100% of MNNG cells became transduced with the tk gene, and the "bystander effect" quite possibly was integral.

These data do suggest that the growth of unmodified MNNG tumors may be slightly inhibited by the administration of GCV; the final maximal diameters of these tumors were 19.5 +/- 1.6 mm for mice receiving GCV versus 23 +/- 2.5 mm for mice receiving saline. Although the error bars overlap slightly for this comparison, it is possible that this trend would be borne out with a greater sample size. This observation is consistent with *in vitro* data, and suggests a low-level of phosphorylation of GCV by cellular kinases resulting in decreased tumor growth.

As was noted, several mice retained a residual inflammatory "scar" for a number of weeks after completion of GCV injections, and the diameter of such scars was included in data analysis. Histological examination of representative scars obtained from mice which made early departures during the experimental course suggested that this tissue was fibrous connective infiltrate, much different in appearance than sections through living tumors. In all but two mice (both in Group 4), this scarlike tissue was eventually cleared away, leaving the original injection site unblemished.

***In Vivo* Bystander Experiment** (figs. 16-18).

MNNG osteosarcoma cells display a potent bystander effect *in vivo*. A mixture containing as little as five percent STK-transduced cells was sufficient to result in complete and lasting tumor in all experimental animals. The bystander effect appears to be enhanced by the mutual presence of MNNG (IL2) and MNNG (STK) cells; when as few as 2.5% of each are co-injected with wild type tumor cells, all mice were cured. Regression of tumor mass typically began on the third day of GCV treatment. The time frame of regression was correlated with the cell mixture received.

Of mice receiving mixtures of MNNG (NV) and MNNG (STK) cells, 97% (34/35) were cured following GCV treatment and many of these mice (29/34) were cured by the 28th day of treatment. Control mice receiving MNNG (NV) and MNNG (STK) cells and no GCV showed a slightly delayed expansion of tumor size compared to mice receiving only MNNG (NV) cells, probably owing to a slower growth rate or decreased tumorigenicity of the STK-transduced cells.

Of mice receiving mixtures of MNNG (IL2) and MNNG (NV) cells, 75% (15/20) were cured following GCV treatment. The cure rate was dependent upon the ratio of cells administered. The IL2-mediated effects began to wane at a ratio of 12.5 : 87.5, (4/5 cured) and dropped sharply at ratio of 5 : 95 (one of five cured). Of mice receiving MNNG (NV) and MNNG (IL2) cells and no GCV, 50% (five of ten) were permanently

cured of tumors. Thus, the ability of IL2-transduced cells to mediate tumor regression increased significantly in groups receiving GCV. Because the IL2 gene product is not known to interact with GCV, this difference was somewhat surprising. No significant difference in tumor growth rates was noted between groups where GCV was the differential parameter. Possibly, GCV acts as a hapten which further activates the immune response in the presence of IL2. Alternatively, soluble IL2 may activate a subpopulation of cells to GCV susceptibility (possibly by altering the transcriptional profile of these cells).

Tumors comprised of MNNG (NV), (STK), and (IL2) were cured with the greatest efficiency compared to other groups. One hundred percent of mice were cured at all levels of wild type tumor content up to a ratio of 95 : 2.5 : 2.5 (N = 41). Even when only one percent of the total tumor cell inoculum was comprised of therapeutic cells, 40% (two of five) mice were permanently cured. Tumors containing fractions of both MNNG (STK) and (IL2) cells along with MNNG (NV) cells grew to markedly smaller volumes than did tumors containing similar fractions of only MNNG (STK) and (NV) cells. For example, the implant of MNNG (NV) and MNNG (STK) cells at a ratio of 87.5 : 12.5 (Group 10) attained an average maximal volume of 66.71 mm^3; when MNNG (NV) cells were mixed with the same number of MNNG (STK) cells along with MNNG (IL2) cells, or a 75 : 12.5 : 12.5 ratio of each (Group 8), the maximal tumor volume attained was over eleven-fold less (5.6 mm^3). Even tumors containing 87.5% wild type cells mixed with 6.25% each MNNG (STK) and MNNG (IL2) (Group 11) grew to an average maximal volume (6.4 mm^3), over ten times less than of the tumors with 87.5% MNNG (NV) and 12.5% MNNG (STK). Histological examination of tumors isolated from control mice suggested no significant difference in appearance between tumors derived of the various cell combinations.

The enhanced *in vivo* tumor-killing capacity of STK and IL2 in combination may result from at least three general mechanisms. First, each gene product may act singly to inhibit tumor cells, and the overall effect would be the sum of each individual contribution. Secondly, there may exist a cooperation between the two components, whereby the composite antitumor effect would greater than the sum of the parts. A third possibility is that a synergy exists between the STK and IL2 gene products, whereby each potentiates the other to more efficient activity; in this case, the overall effect would be multiplicative. Additional research is being pursued to elucidate the operative mechanism.

Overall, tumors containing IL2 cells required significantly less time to disappear. Of all mice which were cured, mice receiving mixtures of MNNG (STK) and (NV) cells (N = 34) took an average of 25.2 +/- 3.4 days to be cured. Mice receiving both MNNG (STK) and (IL2) cells along with (NV) cells (N = 36) were cured after an average of 19 +/- 2.9 days. Responding mice which received MNNG (IL2) and (NV) required an average of only 18.6 +/- 3.7 days to become tumor-free. The enhanced rapidity of ablation of IL2-containing tumors is likely to be related to the heightened immune response induced in these mice. NK activity, which is not depressed in athymic nude mice, would be expected to result in the direct attack of tumor cells, speeding the destructive process. Stimulation of phagocytic cells would increase the clearance rate of dead cells; in the absence of this stimulation, dead cells may linger at the tumor site and make continued contribution to the tumor volume. Also, tumors containing IL2 tended to grow to smaller maximal volume, and a smaller burden would logically be eliminated more quickly.

The presence of IL2-transduced cells in the induced tumors had a significant effect on the growth of the tumor mass. Of the mice that received a $2x10^6$ cell inoculum of only MNNG (IL2) cells, 25% (two of eight) failed to develop any tumor. The other mice showed only minimal growth (average maximal volume of 3.1 mm^3) followed by rapid ablation (six of seven showed no tumor by day 21). Of the three mice receiving a larger inoculum ($5x10^6$) of MNNG (IL2) cells, each displayed small tumor growth (average maximal volume of 5.2 mm^3) followed by rapid ablation (no tumors by day 18). This general effect was noticed both in the presence and absence of GCV. Even when only the inoculum contained only a small fraction of IL2-transduced cells, a significant reduction of tumor growth was observed. When a 95:5 ratio of MNNG (NV) to MNNG (IL2) cells was introduced, the maximal growth was only 13.9% of the growth of MNNG (NV) cells alone (60.3 mm^3 versus 432.8 mm^3 at day 18.)

Among the mice not cured, tumor progression was typically much slower in mice receiving IL2-transduced tumor cells. For example, in comparing control groups 21 (NV75 : STK25) and 23 (NV75 : IL2 25), a significant difference ($p < 0.05$) was seen in the tumor volume. Tumors by day 46 in the IL2-containing group had grown to an average volume over 53 times less than that of the STK-containing group (8.7 mm^3 versus 465.7 mm^3). Moreover, the substitution of 10% MNNG (IL2) tumor cells for wild type MNNG cells resulted in a significant ($p < 0.05$) difference in average maximal volume (13.2 mm^3 versus 576.2 mm^3) and slight arithmetic growth as opposed to

geometric growth. The reduction in tumorigenicity of highly malignant cells by IL2 transduction is phenomenon which has been previously noted.[130] This concept has important implications for the development of autologous vaccines by *ex vivo* manipulation of existing tumors.

The appearance of regrowth in a minority of animals probably occurred due to the persistence of a small (usually nonpalpable), localized congregation of resistant tumor cells. These cells may have been somewhat isolated from responsive cells, or may have represented a dormant subpopulation which escaped eradication by virtue of a slow growth rate. Many of the mice receiving IL2-transduced cells alone showed some temporary regression. This is probably due to the activation of an (NK-mediated) immune response which was not potent enough to cope with the entire tumor mass.

The results reported for this experiment actually represent two protocols, the second of which was begun three weeks after the first. Initially, only those groups which had tumors comprised of at least 12.5% therapeutic cells (as well as the control groups) were undertaken. Groups from which the most interesting results were expected were assigned a greater number (ten or eleven) mice. The overwhelming success within these groups prompted the addition of further groups (13, 14, 15, 18, and 19) which explored the applicability of therapy to tumors comprised of a lower ratio of transduced-to-wild type cells. Due to constraints on time, space and animal availability, these groups contained fewer mice.

Double Tumor Experiment (figs. 19-23)

The design of this experiment approaches a more clinically practical scenario, representing an autologous therapeutic vaccine. In the future, it may be possible to treat patients having diffuse, unapproachable, or metastatic cancers by surgically resecting as much of the tumor mass as possible, then reintroducing tumor cells which had been altered *ex vivo* to target remaining neoplastic foci.

This idea of a "magic bullet" for cancer was first proposed by Erlich over a century ago, and the concept has since undergirded decades of work in the realm of immunotherapy. Approaches such as tumor-antigen specific monoclonal antibodies have been promising in theory but disappointing in practice. Genetic therapy may soon offer the means to specifically target cancer by allowing the body to fully harness its own inherent protective mechanisms. Autologous tumor cells would have the advantage of escaping recognition as foreign intruders, by virtue of the self-specific antigens they

would display. Such cells, introduced systemically, might also be expected to retain their programmed migratory capacity and thus become distributed to the more deeply entrenched sites of metastatic disease. Ideally, this could offer a direct means to affect otherwise inaccessible neoplastic foci, analogous to a cellular Trojan horse. By transducing the cells with genes which would bolster the immune response to allow recognition of the malignancy, or with genes which could confer a susceptibility to other pharmacological agents, a dramatic response might be achieved. This approach has the potential to be more universally applicable and less invasive than direct injection of cells (foreign or autologous) into a single tumor mass.

In this experiment, MNNG (NV) cells were initially implanted subcutaneously. One week later, a second inoculum was introduced at a distance of about one cm from the first tumor; this second implant was derived of transduced MNNG tumor cells which expressed either the tk gene or the IL2 gene. These modified tumor cells could potentially initiate a potent and specific immune response (in the form of expanded natural killer cell activity in athymic mice). Alternately, a soluble "bystander factor" might be released from the STK-transduced cells and communicated to the unmodified cells, rendering them susceptible to GCV.

Of the twelve mice in Group 1, 11 (92%) showed significant ($p < 0.05$) regression (i.e., at least a 50% reduction in tumor volume) as a result of a secondary MNNG (STK) tumor and GCV treatment, three of the mice showed over a 90% reduction in tumor volume. Overall, these mice demonstrated a 60.7% reduction in tumor volume, from an average of 552.5 +/- 258 cubic mm at day 19 to an average of 217.2 +/- 58.9 cubic mm at day 25. Because the mice expressed a variety of response intervals (i.e., some tumors were peaking in size while others were rapidly shrinking, and some were regrowing at the same time that others were regressing), the average of the data is somewhat less dramatic than had all mice responded simultaneously. Generally, though, the average tumor volume continued a pattern of increase during the first six days of GCV treatment, then decreased abruptly during the second six days. In the week following GCV treatment, seven of the ten surviving mice had begun to show regrowth of the primary tumor; by the end of the second week, the tumors other three mice had also begun to regrow. By day 41, all tumors had expanded back to an average volume of 498 +/- 213 cubic mm, a level not significantly different than the average maximal volume prior to treatment. Histological examination showed tumor cells which closely resembled the parental MNNG cell line. Inflammatory infiltrate and debris were visible around the tumor

margin, but surrounding tissue appeared to be non-necrotic and non-neoplastic.

Significant evidence of primary tumor regression was also seen in Group 2, which received a secondary implant containing an equal mixture of MNNG (STK) and MNNG (IL2) cells. The peak average volume of the primary tumor (550 +/- 227 cubic mm) was seen on day 41; by day 49, the average diameter had dropped 60.4% (218 +/- 107 cubic mm). This regression was identified at a later time frame than that seen in Group 1. Actually, nine of 17 (53%) of informative animals did show a major (over 50%) regression in tumor volume during the second half of GCV treatment. Of the early responders, 5 (56%) developed subsequent regrowth as early as day 34. Six of 17 mice (35%) showed a delayed response between day 25 and 41, ostensibly due to the action of IL2. Five of the seventeen mice (29%) showed no significant response at any time.

The two mice that were cured both had developed moderately-sized tumors (25.13 and 26.18 cubic mm) by day seven. Each mouse showed a response during both the GCV period (78.8% and 40%), slight regrowth (50.6% and 34.4%), then complete disappearance of the tumor between days 41-49.

In Group 3, all six mice received a secondary implant of MNNG (IL2) cells. Among these mice, the total average reduction in tumor size was 63.1% (745.9 +/- 356 cubic mm to 275.3 +/- 357 cubic mm); the most drastic decrease (95% of the total) was seen between days 28 and 34. No mouse showed a significant antitumor response during the course of GCV injection. With a larger sample size, this relationship may have been more significant.

Interestingly, two of the twelve mice receiving secondary MNNG (STK) cells still retained a significant tumor mass (10.5 mm^3 and 25.7 mm^3), and a third showed a small scarlike residuum. This may indicate the selection of a resistant population of MNNG (STK) cells, or it may signify differences in response capacity between sibling mice. Among mice in the other treatment groups, secondary tumors (consisting of 50% or 100% MNNG (IL2) cells) dissipated quickly and completely, invariably by day 41.

This data indicates that MNNG (STK) and MNNG (IL2) cells are each able to independently effect a significant regression of an unmodified, previously established MNNG tumor. When used together, the two gene products appear operate with an enhanced effect, allowing some mice to be cured completely. The two vector products appear to act according to discrete time intervals. The STK principle mediates an early effect, within the latter part of GCV treatment (mean impact date = 22). The IL2 principle mediates a delayed effect, clearly after the GCV treatment period is over (mean

impact date = 32). The distinct time frame within which each principle acts corroborates the logical inference that each functions via a separate mechanism. The *in vivo* transduction of wild type cells with the STK gene makes them susceptible to killing during the early period in which the active drug is provided. On the other hand, IL2 is likely to operate both nonspecifically (by inducing a generally enhanced state of immunity) and specifically (by making IL2-secreting tumor cells better recognized targets for the immune response). The delayed effect seen with the introduction of IL2-secreting cells is likely to be related to the amount of time needed to induce a specific immune response, largely dependent upon the generation and mobilization of natural killer cell populations. The two mice that were cured in this experiment appeared to benefit from both mechanisms.

It has been suggested that the two principles may interact synergistically to result in a more efficient tumor ablation. This interaction could enhance the "bystander effect." Historically, it has been shown that IL2, through a positive feedback mechanism, induces an increased expression of IL2-receptors on the surface of the cells it affects. Should uptake of the bystander factor be mediated at least partially through this receptor, better delivery and a more efficacious activity would be expected. Moreover, the presence of IL2 may somehow increase the half-life of the bystander factor, affording it more time to communicate itself to targeted osteosarcoma cells.

Because of the progressive growth in tumor size among the mice receiving a secondary MNNG (BAG) implant, it can be confidently asserted that the anti-tumor response derives from the specific gene encoded by the vector, rather than something inherent in the murine fibroblasts into which the vectors are packaged. Moreover, X-Gal staining of the primary tumors in these animals was wholly negative. This indicates that neither the BAG gene nor appreciable levels of the enzyme are transmitted from cell to cell. It is not surprising that the vector-producing cells have a limited range which the viral particles are able to access. By extension, the response seen by mice in therapeutic groups must occur by some means other than cellular transduction.

Thus, it would seem that the MNNG (STK) cells may be producing some substance which is transmitted over a distance to neighboring tumor cells. However, this factor is not able to deliver itself to a sufficient number of the unmodified cells to mediate a complete and lasting cure. In the presence of IL2, higher efficacy is seen. Changes in tumor morphology provided some insight as to the events taking place. Primary tumors

were generally ovoid and symmetrical during initial growth; in some cases, more pronounced regression was seen in the side proximal to the secondary tumor. Although this observation constitutes a statistically inert perceptual discrimination, it does bring evoke the image of a bystander factor which is either labile or unable to penetrate to the distant side of the tumor mass.

Much attention has recently been afforded this "bystander factor." The phenomenon of tk-negative cells becoming susceptible to GCV along with tk-positive cells has been identified in a number of experimental models, yet at the present time its identity remains elusive. The factor may simply be activated GCV triphosphate which is passed through cellular junctions or taken up fromapoptotic cells. In this case, the bystander effect would be akin to a metabolic cooperation whereby cells, not themselves expressing thymidine kinase, acquire the means of their own destruction through the catabolism of tk-positive cells. Alternatively, the bystander factor may be a distinct macromolecule which is formed in tk-positive cells.

The data here afford only an ambivalent answer to the question of HS-tk/IL2 synergy. *In vitro*, a definitive argument against HS-tk/IL2 synergy for the MNNG cell line is seen; here, the presence of IL2 does not significantly enhance the effect of STK-mediated killing. *In vivo*, both vectors generate a specific anti-tumor response which is enhanced when both components are present. The temporal difference in action is suggestive of two distinct, cooperative mechanisms rather than a unified, synergistic mechanism. The lack of correlation between *in vitro* and *in vivo* data implies that the immune system is of paramount importance to the HS-tk/IL2 interplay.

Paradoxically, recent *in vitro* work suggests that, unlike some other cell lines, MNNG does not produce a soluble bystander factor. For some cell lines, the transfer of media from STK-transduced cells results in a substantial decrease in the proliferation of wild type cells; this has not been found to be the case for MNNG cells. Although this seems to be contradictory, both sets of results could easily be accurate given the differences between *in vivo* and *in vitro* models. In any case, the topic merits further research.

Treatment of Established Osteosarcomas (figs. 24-30).

The treatment of cancers by direct injection of vpc may prove to be a potent application of gene therapy. This approach is especially suited to localized tumors. Clinical trials are currently underway for the treatment of brain cancer. Many patients

with osteosarcoma could be excellent candidates due to the typical course and nature of the disease, especially the metastatic component. The cure rate of current therapies for osteosarcoma are less than ideal, and often entail the inestimable morbidity of losing a limb. Spread of the cancer to the lung or another bone is a significant problem. Successful gene therapy for osteosarcoma could be an enormously satisfying alternative for patients in that it could selectively destroy the metastatic disease and preclude amputative surgery.

The evaluation of this approach in animals is a necessary step in the process of gaining approval for new methodologies in medical practice. In working with bone cancer, the ideal scenario would be the induction and treatment of cancer cells which are localized in the bone. However, nude mice are small and naturally frailer than even normal mice; their long bones are similar in size and durability to dry toothpicks. Moreover, these animals are not especially tolerant of repeated invasive procedures. For these reasons, our treatment model used subcutaneously induced osteosarcomas.

Trial One (fig. 24). In this experiment, 40% of mice (four of ten) receiving an intratumoral implant of STK vpc showed a significant reduction in tumor size; 33% of mice (three of nine) receiving an intratumoral implant of IL2 vpc showed a significant response; 11% of mice (one of nine) receiving combination treatment were cured completely.

Of the responding mice which received STK vpc (Group 1: fig. 25), treatment with GCV appeared to exert an early influence. Between days 14 and 18 (corresponding to days 2-6 of GCV injections), the maximal tumor diameter remained relatively stable, around 15 mm. By day 19 (corresponding to the seventh day of GCV), each responding animal showed a drop in tumor diameter, and daily measurements tracked a downward trend for the next eleven days. In contrast, the tumors in the GCV-negative subgroup continued to grow progressively. The response in this group was ostensibly due to the inhibition of the population of tumor cells which had been transduced with HS-tk .

Of the responding mice which received IL2 vpc (Group 2: fig. 26), a similar plateau in tumor diameter was observed from days 13 to 18, followed by a significant shrinkage (78%) occurring from day 20 to 29. The nonresponding mice which received GCV showed a slower progression of tumor growth compared with the GCV-negative subgroup. Because IL2 and GCV are not known to act upon one another, it was expected that the tumors would show a similar growth pattern regardless of the presence of GCV.

The partial response of some tumors to GCV was somewhat surprising and is probably related to the presence of natural murine kinases.

Of the ten mice that received STK and IL2 vector producer cells (Group 3: fig 27), only one showed a significant reduction in tumor size. The responding mouse was cured of its tumor during the first four days of GCV administration. Although it is difficult to generalize from a single animal, this mouse did have a smaller tumor prior to GCV treatment (the maximal diameter was seven mm, on day 11). This may indicate that a favorable outcome is more likely when a small or slowing growing tumor is treated. Also, due to its smaller tumor, this mouse may have benefited from a better delivery of vpc. The nine nonresponsive mice showed progressive growth, but at a significantly lower level than the mice which did not receive GCV.

Mice in Group 4 were controls for the effect of the vector. The introduction of the ß-galactosidase gene is experimentally null and would not be expected to have any therapeutic effect. Thus, this group was included to show that the response seen with HS-tk and IL2 vpc was a direct consequence of the protein translated from the inserted gene rather than a nonspecific, inherent effect of the murine fibroblastic cells. Moreover, the efficiency of transfer of the BAG gene can readily be determined by X-Gal staining. Mice receiving BAG vpc and GCV did show a significant ($p < 0.05$) reduction in tumor diameter (55.8%) versus mice receiving BAG vpc and no GCV. This is presumably due to low-level phosphorylation of GCV by cellular kinases.

This experiment was actually the first to be attempted, and because of this, the growth parameters of MNNG had not yet been characterized. The original inoculum of $5x10^6$ was twenty-five times the minimal inoculum needed to consistently induce tumor formation; thus, this protocol represented a more formidable treatment challenge than may have been necessary. By day five, when the vector producer cells were injected, the tumors were typically peanut-sized, which is proportionately enormous for a 20 gram animal. Extrapolating from the determination of growth rate *in vitro*, the MNNG cells would have had sufficient time for five replication cycles, meaning that the initial 5 million cells may have expanded to over 160 million cells by the time of vpc injection. Of course, *in vitro* growth cannot be expected to correlate exactly when presented with the added variables of growth in an experimental animal, but these figures are probably a reasonable approximation. A tumor of this mass is likely to have a substantial effect on

the general health of an animal, making it less amenable to treatment and less able to mount a proper immune response.

Furthermore, the injection of 15 million vpc per tumor approaches the limit for the total volume that may safely and effectively be injected without damaging the skin or introducing a cellular embolism into the vasculature (which is enhanced around the tumor). As would be determined subsequently in the first mixture experiment (when MNNG cells were co-injected with STK vpc), at least a one-to-one ratio of tumor cells to vector producer cells is necessary for the therapy to be fully effective. Here, the ratio of MNNG cells to vpc was perhaps as high as ten to one. Moreover, the mixture has the advantage of approximating optimal delivery, whereby tumor cells and vpc are evenly dispersed, increasing the opportunity for *in vivo* transduction. Here, efforts were made to inject the vpc throughout the tumor (two injection tracts were used to access both sides of the tumor mass), but there is no evidence that the therapeutic cells are capable of uniformly infiltrating the tumor. Discrete areas of the tumor which were not breached by the vpc would probably be precluded from transduction and may have been sheltered from the action of the bystander effect as well.

To further compound matters, the tumors grew so rapidly that it became necessary to euthanize many of the animals early in the follow-up period. The delayed activity of IL2 (which seems to have an effect during the fourth week after administration) was not seen. It is possible that the tumors grew too quickly for an IL2-induced immune response to have an impact. Ideally, all mice receiving IL2 vpc would have been monitored for an additional several weeks. By day 24, however, the tumors of nonresponding and control mice were generally too large to allow the experiment to continue.

The next treatment protocol was modified to take the aforementioned factors into account. Unfortunately, a change in corporate policy made it impossible to further utilize the IL2 vpc, which had been obtained under stipulations. Thus, it is not possible to estimate the potential impact these cells may have had under more favorable experimental conditions. Also, after the first treatment experiment, it was decided that three-dimensional measurements would be more informative.

In the second treatment experiment (fig. 29), mice received an initial inoculum of $5x10^5$ cells, which was ten times less than that used previously. Five days later, the tumors generally had maximal diameters between two and three millimeters and were more like grapefruit seeds rather than peanuts in size. Again, to extrapolate from *in vitro*

data, the number of tumor cells present at this point may have been as many as 16 million. An inoculum of 20 million vector producer cells (an increase of 33% from the previous experiment) was injected into the tumors, which may have represented a more compatible ratio of vpc to tumor cells.

During the course of GCV which followed, 15 of 19 mice (78.9%) showed an initial response in the form of a significant reduction in tumor size. One of these animals (5.3%) was cured permanently, while the others eventually developed regrowth. Six mice were given a second injection of STK vpc. The second injection of vpc proved to be very traumatic for the mice. At this point in the experiment, the tumors, having grown for over six weeks, were typically large and highly vascular (especially in those mice not showing an initial response). Mice in the BAG group were handled first. Of the five mice having received a primary inoculum of BAG cells two weeks earlier, four (80%) died in the throes of violent seizures within seconds after the secondary BAG injection. The animals having larger, more vascularized tumors seemed to be the most affected. In several cases, it was possible to observe (through the translucent skin) a traveling difference in color through superficial ascending veins. It is possible that the some of the cells found their way into the blood supply, resulting in a fatal embolism.

With this in mind, three of the STK mice which had large and highly vascular tumors were excluded from a second vpc injection. These mice were regrouped, and were eventually tabulated among the nonresponders. Six of the seven mice receiving a second injection of STK vpc survived the process. These mice, having already undergone a week long course of GCV injections, were visibly more frail, so the dose of GCV was reduced by 17% (from 3 mg/ml bid to 2.5 mg/ml bid) in an effort to keep the mice alive. In spite of this, two of these six mice died during the course of GCV treatment. The four remaining mice did show a significant response ($p < 0.05$) to both STK vpc injections. It is possible that the first treatment course contributed to the selection of a subgroup of MNNG cells which had developed a mechanism to enhance their survival; such means could include a decreased susceptibility to retroviral infection, interference with the retroviral integration process, altered cellular receptors, or improved sequestration and export of GCV or GCV-TP.

Overall, the size differential between the tumors in the first and second treatment attempts seemed to have a positive impact in the post-therapy prognosis. Accordingly, the third protocol was designed to intercept the tumors early in the growth period, when neoplasms were relatively small and prior to the onset of log phase growth. For this

experiment, twenty-one mice received $2.5x10^5$ MNNG (NV) cells subcutaneously. Six days later, small tumors had begun to grow in each mouse, averaging less than two cubic millimeters per animal. These tumors were considerably smaller in this trial than they had been at the corresponding point in the previous experiment; where the tumors in the second treatment protocol were comparable in size to grapefruit seeds at day six, here the tumors were analogous to pinheads. At this point, STK vpc (1.5×10^7) were introduced as a single injection; the cells flooded the tumor site and raised the neoplastic foci (which remained visibly distinct) onto a slight plateau.

The number of tumor cells present at the time of the STK vpc injection may be roughly estimated at 8 million. Of course, this assumes an *in vivo* generation time of 24 hours. Actually, tumor cells given at this dilution may require more time to move from lag phase to log phase growth, so fewer cells may have been present. Indeed, the relatively small size of these tumors supports this supposition. Thus, it is likely that the ratio of STK vpc to MNNG cells was better than two to one in this case. Moreover, judging from appearance, the cells were well-delivered, as the entire tumor was in each case engulfed.

As before, the responding animals generally exhibited the most pronounced reductive effect during the second half of GCV injection. This time interval indicates either a lag between the GCV-mediated termination of DNA synthesis and the eventual death of the cells, a requisite threshold to GCV exposure, or the average time required for the incorporation of activated GCV. Repeated injection of STK vpc might have further improved the response.

Among the three animals which were cured in the third trial, the average tumor volume remained relatively low throughout the experiment. In contrast, the tumor volumes for the six partial responders were significantly higher than the volumes for the nonresponders between days seven and 14. This suggests a relationship between rapid tumor growth and the transduction efficiency of the STK vector. Tautologically, a rapidly growing mass of cells will be undergoing DNA replication and mitotic division at a faster rate than a slower growing mass; these conditions would make more likely the infection by retroviral particles, and would also increase the incorporation frequency. A population of cells with a higher transduction efficiency (or, with a higher multiplicity of infection) would subsequently be expected to phosphorylate GCV with more zeal. As evidenced by X-Gal staining of tumors injected with BAG vpc, the transduction efficiency can vary greatly among similar tumors. Moreover, as the delivery of vpc

seemed to be optimal in this trial, the final outcome of treatment may depend exquisitely on the dynamics of transduction.

Overall, the response rate of 50% and the cure rate of up to 15% seen in these experiments are similar or better to the figures achieved by conventional strategies used in the treatment of osteosarcoma. Thus, this work justifies further research in the area, which should eventually include clinical gene therapy trials for patients with osteosarcoma.

Limitations in the efficiency of gene transfer are most likely to be at the crux of the marginal success rate. Accordingly, future advances in the science of gene therapy must include efforts to improve the gene transfer process. As has been described, the use of retroviruses represents an effective way to stably convey genetic material, with efficiencies exceeding other means by tenfold or better. Under the conditions of these experiments, the transduction efficiency with the BAG gene varied from less than one percent to over 14%, with an average of about six percent (table 15). These figures give a basis for the transduction expectations for the STK gene. Actually, the transduction efficiency with the STK gene might be expected to be somewhat higher in that these vpc are reported to generate a higher titer than are the BAG cells. Our experiments have indicated that a population of as little as five percent STK-transduced cells is sufficient to mediate a significant response. Of course, in the context of the treatment experiments, the transduced cells were not uniformly distributed throughout the tumor.

In this series of experiments, attempts were made to improve transduction efficiency both through improving the vpc to tumor cell ratio and by using repeated injections of vpc. Each of these approaches enjoyed some success, but it is likely that better results would be abtained with more effiecient transduction. In the future, the strategy of multiple vectors (either simultaneous introduction of multiple genes in single vectors or a single wector containing an operon of several genes driven by the same promotor) might contribute to a positive response.

Attempts to develop a lung metastatic model

The process of metastasis is not random, but is generally thought to represent a highly selective event[169] which favors the survival of a subpopulation of variant cells which preexist in the parental neoplasm.[170] This process consists of a requisite series of sequential steps that potentially metastatic cells must follow. These steps include: invasion by metastatic cells, embolization, persistence in the circulation, arrest in a

distant capillary bed, and extravasation and multiplication in the parenchyma of a receptive organ.[10] The interruption of any of these steps would preclude the development of metastasis.

Thus, spontaneous metastasis in an experimental model may be viewed as a highly unlikely event. The fulfillment of all the requirements for metastasis is compounded by factors relating to nude mice. Natural killer cells (which nude mice possess in high levels) are reported to be particularly adept in eradicating tumor emboli from the circulation.[171] Also, young (two to six weeks old) nude mice are reported to be more susceptible to metastasis, while neoplasms only rarely metastasize in adult nude mice,[172] which is likely to be related to the age-specific development of immunity. The mice used throughout these experiments were initially six to eight weeks old, and thus might be expected to have a significant level of immune responsiveness. Finally, the metastatic potential of human neoplasms can vary with the site of implantation to nude mice.[10]

Injections into the tail-vein of nude mice were done in an effort to develop a metastatic model. There is an urgent need for such models, as much of the mortality of patients with osteosarcoma stems from uncontrollable spread of malignancy, most commonly to the lungs. Here, cells introduced into venous circulation would be expected to enter the inferior vena cava en route to the heart. Cells reaching the right ventricle from the right atria would pass into the pulmonary artery and then through the branching network of vessels perfusing the lung. At this point, some cells might be expected to breech the capillary endothelium and enter the interstitium to colonize lung tissue. If conditions are right, a tumor foci might develop and expand. Injection of various concentrations of MNNG cells to the tail veins of over thirty mice failed to result in any tumor development beyond the caudal appendage. Ostensibly, a weak link in the chain of the metastatic process was exploited; the tumor cells may have been recognized and destroyed in the blood system by natural defense mechanisms, or the cells may not have had the capability to leave the vasculature. Alternatively, the specific population of MNNG cells introduced may not have contained metastatic-competent variants.

Metastasis of osteosarcoma cells was eventually achieved through a different approach. The fifth osteosarcoma cell line (MLM, for MNNG Lung Metastasis) was derived from a lung tumor induced by metastasis of MNNG cells in one of ten mice which received an intratibial implant. In this animal, the right lung harbored a large

tumor mass, which was excised; one half was fixed for histology, and the other half was digested and grown in culture to a permanent cell line.

Subcutaneous introduction of the MLM cells ($2x10^6$) resulted in tumor development in each of five mice. Unfortunately, further *in vivo* experimentation with the MLM line (including intravenous and intraosseous injection to two groups of ten mice each) shows that the MLM cell line has no increased propensity for metastasis to the lung. However, this experience was instructive in that it offered evidence for the practicality of removing osteosarcoma metastases and altering the cells *ex vivo* with therapeutic intent. Efforts to develop a pulmonary metastatic model are ongoing.

CONCLUSIONS

These experiments have demonstrated that gene therapy has the potential for significant therapeutic intervention in the management of osteosarcoma. Each of the five osteosarcoma cell lines studied, including a novel line derived from a lung metastasis, were stably transduced with each gene of interest. Transduction with the HS-tk gene conferred a strong sensitivity to GCV. A potent bystander effect was demonstrated *in vitro* for each line. The *in vivo* studies support the existence of a soluble bystander factor. The HS-tk and IL2 gene products may interact to produce a heightened anti-tumor response. The safety and efficacy for utilizing gene therapy for the treatment of established tumors approached or exceeded success rates for traditional modes of managing osteosarcomas in patients. In the future, gene therapy may be used as one arm of a combined approach to cancer therapy.

A fundamental refinement to this approach to genetic therapy may be necessary to achieve optimal success in the realm of gene transfer. Improvement of the estimated *in vivo* transduction efficiency will likely result in greater overall efficacy. The development of vpc which generate retroviral particles at a higher titer is a logical step, and one which is currently being pursued. Another idea is to prime the target cells in such a way as to enhance the likelihood of retroviral transduction. For example, the polycation protamine has been shown *in vitro* to improve transduction by altering the surface charge profile of target cells. A similar approach could be taken *in vivo* by co-injecting an augmenting compound along with vpc. Efficacy might be further enhanced by the use of vpc lines containing more than one therapeutic gene in the retroviral construct. A cell-free means to deliver retroviruses is also under investigation.

Approaches other than retroviral transduction may also prove to be important and feasible in the future. The use of non-integrative viral vectors (such as adenovirus and vaccinia virus) is currently being explored, as are various other vector-based systems. Improvement of direct methods of DNA transfer (such as liposomes and electroporation) may also play a role. In any event, the complete realization of the full potential of gene therapy will be achieved through ingenuity and creativity as well as hard work. With these components in place, the future outlook for people with osteosarcoma and other aliments is becoming ever brighter.

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